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In this paper, we report for the first time, the synthesis of NPY receptor antagonists using a novel strategy based on screening of peptide analog mixtures. In the synthesis of these receptor antagonists, a series of NPY analog mixtures was first synthesized using a solid-phase synthetic technique. The antagonist-containing mixture was then identified by screening these analog mixtures using a bioassay, and two potent NPY receptor antagonists, designated PYX-1 and PYX-2, were isolated from this mixture. This analog mixture-screening strategy may be used as a general method in developing receptor antagonists for many other peptides.

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Edited by Janet M. Allen and James I. Koenig

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CENTRAL AND PERIPHERAL SIGNIFICANCE OF NEUROPEPTIDE Y AND ITS RELATED PEPTIDES[a]

Editors and Conference Chairs
JANET M. ALLEN AND JAMES I. KOENIG

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Preface

In 1982, Drs. K. Tatemoto and V. Mutt first published the isolation and characterization of neuropeptide Y (NPY). They could not have anticipated the enthusiastic interest in NPY that has evolved over the last eight years or realized how their discovery would influence research workers in diverse fields of neuroscience, basic chemistry, cardiovascular physiology and gastroenterology. NPY consists of 36 amino acids being characterized by an amino terminal tyrosine residue and a carboxy terminal tyrosine amide moiety. As tyrosine is represented by Y in the single letter nomenclature for amino acids, it is these tyrosines that are denoted by Y in the name. In their original description of NPY, Tatemoto and Mutt drew attention to the homology of this newly discovered peptide to the pancreatic polypeptide family of peptides, namely pancreatic polypeptide and peptide YY (PYY). Subsequently, the cDNA and gene encoding NPY were isolated and studies of the regulation of NPY gene expression as well as the physiology of NPY progressed. As an indication of the importance of this family of peptides in biological systems, well over 1000 papers have been published on NPY and its related peptides in terms of the chemistry, physiology, pharmacology and anatomy.

Although a meeting on NPY took place two years ago, the current meeting sponsored by the New York Academy of Sciences represents the first open meeting on the NPY family of peptides. Open communications by young investigators were encouraged and presented in parallel with the more traditional review talks. However, due to the ubiquitous nature of NPY thoughout the body, the scope of the meeting had to be curtailed to keep it within a reasonable time span. We attempted to highlight several areas of NPY research, including an in-depth discussion of the structure of the native NPY molecule, which we hope will serve as a guide to aid in the development of analogues to assess the critical feature of the molecule essential for receptor binding and signal transduction. Emphasis was also placed on the molecular mechanisms regulating the expression of the genes encoding the peptides. Since NPY is concentrated in tissues affecting cardiovascular performance, attention was directed to the significance of NPY in regulating cardiac and renal function. Finally, much of our present knowledge of NPY has been derived from studies of NPY in neuronal systems. Presentations on the anatomy and physiology of NPY in higher brain centers are followed by an in-depth review of the function of NPY in the hypothalamus, concentrating on feeding, reproduction and pituitary function. The overall goal of the conference was to integrate classical neuropeptide studies with modern approaches to the study of these substances, so that the most exciting areas of interest could be identified and explored.

We would like to extend our gratitude to the New York Academy of Sciences, and specifically to Ms. Renee Wilkerson and Ms Geraldine Busacco, for their efforts in organizing this conference. We also would like to acknowledge the contributions of all the participants, both invited and voluntary, for without excellent presentations and someone to listen to them, there would not have been a conference. Finally, we express our appreciation to the government agencies and companies for their support of this meeting; hopefully, it was a worthwhile investment.

Janet M. Allen
James I. Koenig

PART I. NEUROPEPTIDE Y: STRUCTURE AND FUNCTION

Neuropeptide Y and Its Receptor Antagonists

Use of an Analog Mixture-Screening Strategy

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Many of the advances in our knowledge of neuropeptide Y (NPY) and its related peptides have resulted from the application of new research strategies. For example, a novel strategy of searching for unknown peptides that possess the C-terminal amide structure led to the discovery of both NPY[1] and its related peptide, PYY[2], a decade ago. Subsequent studies by a number of groups employing a variety of new biological, anatomical, and biochemical approaches have elucidated many important regulatory functions of these peptides.[3-7]

In this paper, we report for the first time, the synthesis of NPY receptor antagonists using a novel strategy based on screening of peptide analog mixtures. In the synthesis of these receptor antagonists, a series of NPY analog mixtures was first synthesized using a solid-phase synthetic technique. The antagonist-containing mixture was then identified by screening these analog mixtures using a bioassay, and two potent NPY receptor antagonists, designated PYX-1 and PYX-2, were isolated from this mixture. This analog mixture-screening strategy may be used as a general method in developing receptor antagonists for many other peptides.

Neuropeptide Y: Discovery

With the accumulation of knowledge about the structures of many peptide hormones and neuropeptides, it has become evident that these peptides are produced from their precursor proteins by posttranslational processing.[8] The processing of peptides frequently includes unique modifications of the peptide molecules such as phosphorylation, sulfation, acetylation, pyroglutamation and C-terminal amidation. C-terminal amidation is known only to occur in the structures of neuropeptides and peptide hormones in mammalian tissues. This modification can therefore be exploited as a chemical marker to detect neuropeptides and peptide hormones.[9]

In 1973, we initiated a project to develop a chemical assay for secretin and cholecystokinin. Our original intention in developing such a chemical assay was to replace laborious bioassays used during the purification of secretin and cholecystokinin from the intestine. Since these peptide hormones contain the C-terminal amide structure, we proposed a novel chemical assay for these hormones.[10] This chemical method detected the C-terminal amino acid amide which was cleaved off enzymatically and converted to the fluorescent dansyl derivative, then selectively separated and identified. When porcine intestinal extracts were subjected to this assay, it was found unexpectedly, that the extracts contained several previously unknown peptides with the C-terminal amide structure.[10] This finding led to the isolation of a series of novel intestinal peptides

including PHI-27[11,12] and PYY.[2,11] Although these peptides were isolated without any prior knowledge of their biological activities, each of the isolated peptide amides was later found to be biologically active with important neural and/or hormonal properties.[9] Based upon these findings, it was proposed that the isolation of unknown peptides with the C-terminal amide structure would result in the finding of new neuroactive and hormonally active peptides.[11]

The search for new peptides was then extended to the brain where a novel neuropeptide having a C-terminal tyrosine amide was isolated using this chemical assay.[1] The 36 amino acid peptide was named neuropeptide Y (Y = tryosine), or in short, NPY. Subsequent studies have shown that this neuropeptide is a major regulatory peptide in both the central and peripheral nervous systems.[3–7]

Neuropeptide Y: Structure and Function

The primary structure of NPY isolated from porcine brain[13] is shown in Figure 1. NPY was also isolated from other species including human,[14] rat,[15] guinea-pig, rabbit,[16] cow,[7] and sheep.[17] These studies suggest that the NPY structure has been well conserved during evolution. NPY has structural similarities to both PYY and pancreatic polypeptide.[13] The tertiary structures of these peptides also have been shown to be similar.[18,19] The structures of cDNAs encoding NPY mRNAs[18,20] and clones encoding NPY genes[21,22] have been elucidated and the primary structures of the precursors of human and rat NPY have been predicted from these studies.

The localization of NPY has been studied using histochemical and immunoassay techniques. NPY is widely distributed throughout the central and peripheral nervous systems. NPY is distributed in nerve fibers of the brain,[23] spinal cord,[24] blood vessels,[25] heart,[26] intestine, stomach, pancreas, genital tract,[25] lung,[27] spleen,[28] adrenal medulla,[29] and other organs.[25] NPY has been shown to coexist with catecholamines in some neurons.[25,30] Receptor binding studies have indicated the presence of NPY binding sites in the brain,[31,32] spleen,[33] kidney,[34] and vas deferens.[35]

Biological functions of NPY have been extensively studied using various *in vivo* and *in vitro* models. NPY induces strong vasoconstrictor actions,[36] inhibits the release of noradrenaline at the presynaptic level[37] and potentiates noradrenaline-evoked vasoconstriction at the postsynaptic level.[38] Therefore, NPY may play important roles in sympathetic vascular control.[39] NPY strongly inhibits intestinal motility[40] and also modulates

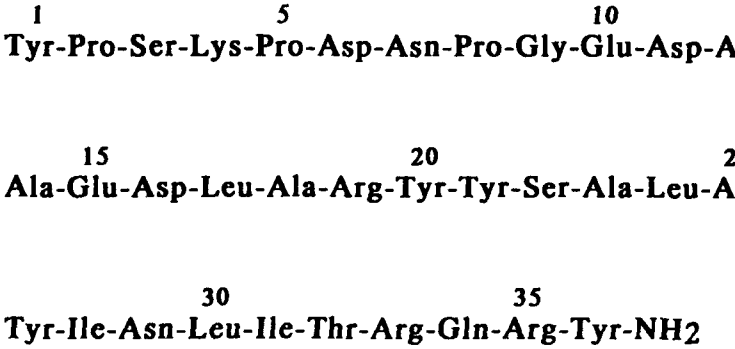

FIGURE 1. Primary structure of neuropeptide Y (porcine).

epithelial ion transport in the intestine,[41] suggesting physiological roles of NPY in gut functions. NPY also inhibits glucose-stimulated insulin secretion[42] and exhibits natriuretic properties.[43] Central administration of NPY induces hypotension and bradypnea.[44] This peptide may therefore be involved in the central control of cardiovascular and respiratory functions. A dramatic increase in food intake upon central NPY administration has been reported,[45-47] suggesting that NPY may play an important role in feeding behavior. NPY injection changes the plasma concentrations of leuteinizing hormone,[48,49] growth hormone, prolactin, thyrotropin,[44] gonadotropin-releasing hormone,[50] ACTH,[51] and vasopressin.[51,52] NPY may therefore play significant roles in the secretion of these hormones. NPY injection into the suprachiasmatic region of the hypothalamus induces a shift in the circadian rhythm.[53] NPY may also play a role in sexual behavior since intraventricular injection of NPY suppresses copulatory behaviors.[54]

A General Procedure for Synthesis of Receptor Antagonists

The search for receptor antagonists of peptide hormones and neuropeptides is important toward understanding the mechanisms of actions, elucidating structure-function relationships, and introducing new pharmacological drugs. However, the design and synthesis of receptor antagonists has proven a difficult and time-consuming task. Despite attempts at applying structural analysis and computer imaging technology, no efficient general method for designing receptor antagonists has been described.

In the present paper, a general strategy for searching peptide receptor antagonists is proposed (FIG. 2). Using this strategy, the synthesis of peptide receptor antagonists can be achieved in a short time. This new strategy begins with the design and solid-phase synthesis of peptide analog mixtures containing hundreds of antagonist candidates. There are many ways of generating such peptide analog mixtures. For example, an analog mixture with various amino acid substitutions can be synthesized by dividing the solid-phase resin into several portions at desired positions during the synthesis, coupling a different amino acid to each portion, and then recombining the portions for further coupling steps. An analog mixture with various chain lengths can similarly be synthesized by withdrawing portions of the resin at desired positions, and recombining the portions for cleavage from the resin.

After the synthesis of a series of analog mixtures, bioassays of competitive receptor assays are used to screen the mixtures for antagonist activity. The individual receptor

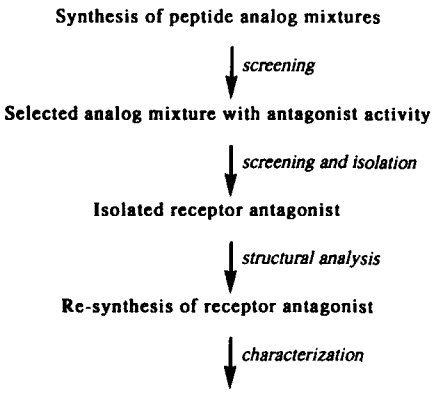

FIGURE 2. Analog mixture-screening strategy for developing receptor antagonists.

antagonists are isolated from the mixtures exhibiting antagonist activities using HPLC and other separation techniques, in a manner similar to the isolation of natural peptides from tissue extracts. The primary structures of the isolated antagonists are then determined, and peptides identical to the isolated antagonists are synthesized for further study.

This approach allows large numbers of peptide analogs to be synthesized and screened in a simple and efficient manner. Moreover, studies using this strategy may reveal the structural requirements for receptor antagonists which could then be used to design and synthesize more potent antagonists. The analog mixture-screening strategy may have wide applications in the development of receptor agonists and antagonists, and might also be applied to the screening of pharmaceutical drugs, as well.

Synthesis of NPY Receptor Antagonists

The novel analog mixture-screening method was successfully applied for the synthesis of potent NPY receptor antagonists. The study began with the synthesis of a series of the NPY analogs each containing one D-amino acid substitution at the C-terminal region of the NPY molecule, since the C-terminal region is known to be essential for receptor binding.[55] The NPY agonist activity of these analogs was then examined by measuring the increase of intracellular calcium in human erythroleukemia cells[56] and the analogs having little or no agonist activity were selected from these analogs. The selected analogs were further subjected to a NPY antagonist assay, which measured inhibition of the NPY-stimulated increase in intracellular calcium in human erythroleukemia cells. Although none of the analogs was found to exhibit antagonist activity, a series of analog mixtures were synthesized based upon the structures of these selected analogs. After the screening of these analog mixtures, a [D-Thr^{32}] NPY analog mixture was found to have antagonist activity. Two NPY receptor antagonists, designated PYX-1 and PYX-2, were isolated from this mixture after three consecutive HPLC steps. Structural analysis revealed that PYX-1 is a decapeptide amide with a modified amino acid at the N-terminus and PYX-2 is a decapeptide amide with modified amino acids at both N- and C-termini (FIG. 3). PYX-1 corresponded to Ac-[3-(2-6-dichlorobenzyl)-Tyr^{27}, D-Thr^{32}] NPY 27–36, and PYX-2, to Ac-[3-(2-6-dichlorobenzyl)-Tyr^{27,36}, D-Thr^{32}] NPY 27–36 (in preparation). It seems that a D-substitution of threonine at position 32 and a modification of tyrosine at position 27 converts the C-terminal decapeptide amide of NPY to a receptor antagonist. PYX-1 inhibited the NPY action in releasing intracellular calcium in human erythroleu-

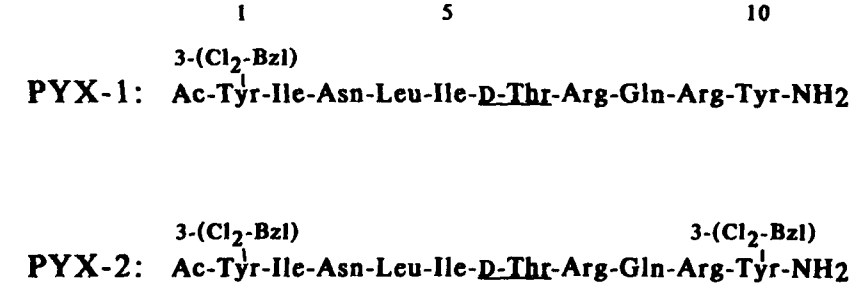

FIGURE 3. The structures of NPY receptor antagonists. 3-(Cl_2-Bzl): 3-(2-6-dichlorobenzyl), -NH_2: amide, Ac-: acetyl-.

kemia cells at a range of 10^{-6} to 10^{-7} M, while PYX-2 at 10^{-7} to 10^{-8} M, indicating that PYX-2 is a more potent antagonist than PYX-1. Therefore, an additional modification at the C-terminus seems to potentiate antagonist activity by affecting the binding to NPY receptors. The results of binding studies indicated that these analogs specifically inhibited the bindings of ^{3}H-labeled NPY to its receptors.

The potential effects of such NPY receptor antagonists on food intake, blood pressure, hormone secretion, pancreatic secretion, gastrointestinal motility, and circadian rhythmicity might have important pharmacological and clinical applications. Further studies of NPY antagonists might lead to the identification of drugs for treatment of obesity, hypertension, sexual dysfunction and sleep disorders.

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Neuropeptide Y Receptor Subtypes, Y1 and Y2

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INTRODUCTION

Neuropeptide Y (NPY) is an amidated 36 amino acid peptide[1] with a wide distribution in the central and peripheral nervous system.[2–5] Accordingly, its effects are many and varied. NPY forms a family of peptides together with peptide YY (PYY; approximately 70% homology) and pancreatic polypeptide (PP; approximately 50% homology). PYY has been found to occur in endocrine cells of the distal intestine[6] and neurons of the brainstem, albeit in much lower concentrations than NPY.[7,8] PP, not being as well conserved between species as NPY and PYY (*e.g.*, REF. 5 and 11), is produced by a special type of endocrine cell of the pancreas.[9] NPY and PYY are both extremely bioactive (cf. TABLE 1 and 2), whereas PP is generally much less active. In our experimental systems, the activity of NPY and PYY is principally always the same; however, their relative potency may vary slightly, PYY often being more active. Therefore, when various effects of NPY are discussed below, it should be kept in mind that exogenous PYY is presumably active as well. This apparent interchangeability of NPY and PYY is also the main reason why we chose to introduce the nomenclature Y1 and Y2 as defined below. Nevertheless, certain concentrations of iodinated NPY and PYY may selectively label different sites in rat brain.[10]

Very much like "classical" neurotransmitters, NPY may exert a wide range of effects on peripheral (TABLE 1) and central (TABLE 2) targets as reviewed previously.[4,5,11] Some of these effects may be exerted by NPY *per se*, whereas others occur as a result of modulatory interactions with other agents (cf. FIG. 1 and below). In any event, it is likely that NPY (and PYY) act at specific receptors. Radioreceptor studies with iodinated or tritiated NPY have detected specific NPY binding sites in brain (*e.g.*, REF. 12–19), vasculature,[20,21] heart,[22] spleen[23] and uvea.[24] Binding sites for ^{125}I-PYY have been described in brain[10,16,25] and intestine.[26,27] Autoradiographic studies of brain using ^{125}I-

TABLE 1. Peripheral Neuropeptide Y Target Tissues[a]

1. Blood vessels (vasoconstriction)	9. Islet β cells (insulin)
2. Heart (chronotropy; inotropy)	10. Thyroid gland
3. Airways	11. Sympathetic nerves
4. Large intestine (motility)	12. Parasympathetic nerves
5. Small intestine (secretion & motility)	13. Sensory nerves
6. Kidney (collecting tubule)	14. Endothelial cells
7. Juxtaglomerular cells (renin)	15. Platelets
8. Atrial cardiocytes (ANF)	16. Mast cells

[a]For references, see REFS. 5, 11, this paper and other papers of this volume.

$NPY^{10,28-32}$ or $^{125}I\text{-}PYY^{10,16}$ have established similar receptor kinetics as in brain homogenates, whereas a few discrepancies in terms of distribution (homogenates *vs* autoradiography) have been noted (*e.g.*, REF. 11).

NPY and the Sympathetic Nervous System

NPY (or PYY) given exogenously has been found to affect both pre- and postjunctional (-"synaptic") mechanisms, presumably by activating receptors on nerve terminals (usually associated with inhibition of neurotransmitter release) and on effector cells (such as nerve and smooth muscle cells), respectively. Many studies have been concerned with the actions of NPY at sympathetic neuroeffector junctions. Obviously, these studies were stimulated by observations of the prevalence of the peptide in sympathetic, notably perivascular, nerves and its coexistence with norepinephrine (NE) in these nerves (*e.g.*, REF. 3). Briefly, we and others have previously demonstrated three effects of NPY at sympathetic neuroeffector junctions: 1) a direct postjunctional response, *e.g.*, vasoconstriction manifested in certain vascular beds; 2) a postjunctional potentiating effect on NE-evoked vasoconstriction; and 3) a prejunctional suppression of stimulated NE release (cf. REFS. 5, 33–35 and FIG. 1). The two latter phenomena are probably reciprocal; thus NE may affect NPY mechanisms similarly.[5,21] Recently, we have argued that the vascular

TABLE 2. Effects Associated with Centrally Applied Neuropeptide Y[a]

I. *Pituitary hormone release*	II. *Behavioral*
1. Growth hormone	1. Eating
2. Thyrotropin	2. Drinking
3. Luteinizing hormone	3. Circadian rhythms
4. Prolactin	4. Sedation/anxiolysis
5. Vasopressin	5. EEG
6. Adrenocorticotropin	6. Sexual activity
	7. Memory
III. *"Autonomic"*	IV. *"Neurochemical"*
1. Blood pressure	1. Norepinephrine turnover
2. Heart rate	2. Dopamine turnover
3. Respiration	3. α_2-adrenoceptors
4. Thermoregulation	4. Melatonin synthesis
5. Gastric acid	5. Norepinephrine release
6. Circulating insulin	6. Glutamate release
7. Urinary bladder	

[a]For references, see REFS. 4, 5, 11, this paper and other papers of this volume.

cooperativitiy of NPY and NE *in vivo* as well as *in vitro* in large part is accounted for by theshold synergism phenomena rather than receptor-receptor interactions and that, when present, α-adrenoceptor reserve prevents the lowering of the NE response threshold by NPY.[21] Hence, it is likely that that NPY receptors responsible for potentiating NE-evoked vasoconstriction in principle could be identical to the NPY receptors associated with direct vasoconstriction. Prejunctionally, it is conceivable that NE and NPY release is "cross-regulated" by feedback stimulation of "autoreceptors" for both messengers and that they activate similar, if not identical, mechanisms in the terminals leading to inhibition of stimulated NE as well as NPY release. The three actions of NPY at sympathetic neuroeffector junctions are illustrated schematically in FIG. 1. The top right panel of FIG. 1 illustrates also that responses to NPY, when compared to NE, generally are slower in onset and more long-lasting; this is exemplified by recordings of pressor responses to NE or NPY in conscious rats. Obviously, however, blood pressure measurements following systemically applied NPY will reflect actions of the peptide on pre- as well as postjunctional receptors in numerous vascular beds; for this reason we and others have studied the actions of NPY on various *in vitro* preparations (see below), and the latter were also used to obtain preliminary evidence for heterogeneity among NPY receptors (see REFS. 5,33–35 and below). The complexity of the *in vivo* setting was further emphasized by our recent findings that high doses of NPY as well as certain C-terminal NPY fragments have the capacity to reduce blood pressure (REFS. 36 and 37 and below). Finally, it should be noted that NPY (and PYY) far from always can be demonstrated to exert pre- *and* postjunctional effects in a given sympathetic target.

We have suggested that the vasoconstrictor *activity* of NPY becomes more important during situations of high sympathetic nerve activity.[5,21] During resting conditions, little NPY is released[38–40] and sympathetic nerve activity is largely reflected by adrenoceptor activation. Increased sympathetic nerve activity is accompanied by both adrenergic desensitization and increased NPY release.[21,40] This may result in a dual effect of NPY. First, NPY may restore the lost responsiveness to NE and, second, NPY *per se* may become a more efficacious vasoconstrictor agent.[21]

NPY Receptor Heterogeneity at Sympathetic Neuroeffector Junctions

A few years ago, in order to compare the effects of NPY with those of chemically related peptides, we characterized some NPY-responsive smooth muscle preparations designed to represent each of the three principally different effects of NPY at sympathetic targets (FIG. 1 and REFS. 5, 33–35): 1) direct postjunctional effects of NPY were studied on isolated blood vessels, for example, guinea pig iliac vein and cat middle cerebral artery; 2) the postjunctional potentiating effect of NPY on NE- (or histamine-) evoked vasoconstriction was studied on other isolated blood vessels, such as rabbit femoral artery or vein, or rabbit pulmonary artery; and 3) the prejunctional suppressive effect on neurotransmitter release was usually studied on rat vas deferens (cf. REFS. 5, 33–35). In all three types of assay, NPY and PYY were both active while PP displayed much weaker activity. Additionally, and in agreement with REF. 41, we found that desamido-NPY (NPY free acid) was virtually inactive in every assay. Therefore, we have frequently used desamido-NPY as a negative control in our work, *e.g.*, when microinjecting NPY into brain.[42] It has also been found that desamido-NPY is several orders of magnitude less potent than NPY in receptor binding studies (REF. 13 and TABLE 4).

To our surprise, however, we found that long C-terminal fragments of NPY and PYY, while being essentially inactive in the assays for postjunctional activity (*per se*-effect as well as potentiation), retained substantial efficacy prejunctionally (inhibition of transmitter release). Some of these data are summarized schematically in TABLE 3. On the basis

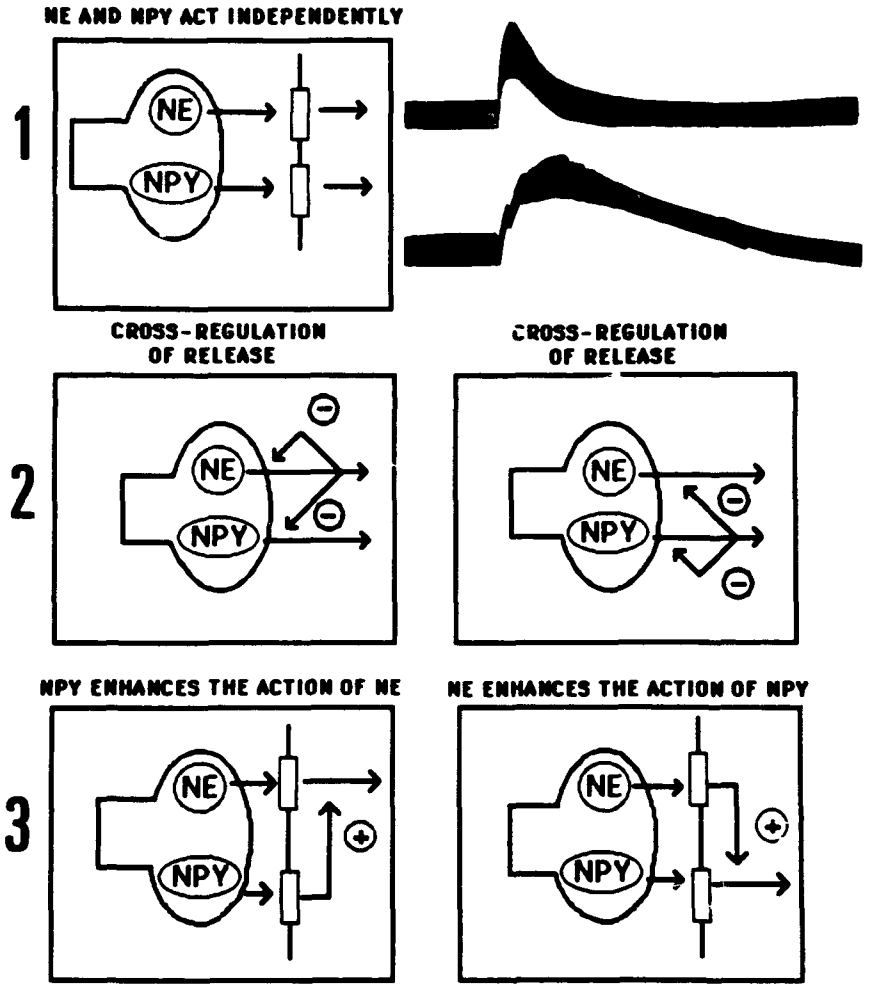

FIGURE 1. Schematic illustration of the three principally different types of effect of NPY (or PYY) at sympathetic neuroeffector junctions: 1) NE and NPY act independently, however *(right panel)* characteristics of response may differ (exemplified by vasopressor effects); 2) Reciprocal regulation of release; 3) Reciprocal enhancement at the level of vascular smooth muscle. See text for further information.

of the selective prejunctional effect of the long C-terminal NPY (or PYY) fragments, it was proposed that NPY/PYY receptor subtypes may exist.[5,33–35] The nomenclature Y1 and Y2 was introduced to denominate the receptor that required the whole NPY (or PYY) molecule for activation (Y1), and the other receptor that was selectively stimulated by the long C-terminal NPY (or PYY) fragments (Y2), respectively.[5,35] In our neuromuscular preparations, the postjunctional receptors were thus (predominantly) of the Y1-subtype, while the prejunctional receptors were of the Y2-subtype. However, more recent experiments conducted by ourselves and others have indicated that, while the Y1-receptor

indeed is the major vascular NPY (PYY) receptor, the Y2-receptor can also occur on (postjunctional) vascular smooth muscle (cf. below). There seems to exist an analogy to α_2-adrenoceptors which were originally thought to be exclusively prejunctional but later were found on vasculature as well.

NPY Receptor Heterogeneity in Other Experimental Systems; Evidence from Use of NPY and PYY Analogs

The Y1-Receptor

Until the very recent development by Schwartz and collaborators[43] of a Y1-selective agonist (by a single critical amino acid substitution, of glutamine to the rigid residue, proline, in position 34), the Y1-receptor was only defined in a negative manner, *i.e.*, as a NPY (and/or PYY) receptor with poor affinity for long C-terminal NPY (or PYY) fragments (*e.g.*, REFS. 5, 33–35, 44). Nevertheless, even in the absence of the extremely useful tool, [Pro^{34}]NPY, or variants thereof, "negative" data were accumulated in support of several possible functions of the Y1-receptor. For example, it was argued that the Y1-receptor represents the major NPY/PYY receptor responsible for the blood pressure increments evoked by systemic peptide administration.[5,33–35,45] This suggestion that the Y1-receptor is the predominating vascular receptor was corroborated by the use of [Leu^{31}, Pro^{34}]NPY and [Ile^{31},Pro^{34}]NPY which were even more potent pressor agents than NPY itself (43 and unpublished data). However, it should be emphasized that the Y2-receptor may well occur in certain vascular beds (*e.g.*, cardiac) but that this subtype probably contributes less to systemic blood pressure increments evoked by NPY (see below also).

From studies on the effects of NPY, and natural fragments, evoked by intracerebroventricular (ICV) administration, functional evidence for central NPY receptor heterogeneity has emerged. Being interested in the sedative/anxiolytic properties of NPY, we compared the effects of NPY 13–36 to those of the parent molecule.[46] To our surprise, we found that the fragment, instead of being sedative (suppressing activity) like NPY, in fact increased behavioral activity (REF. 46 and FIG. 2). Therefore, we proposed that the behavioral/anxiolytic effect of NPY might be Y1-receptor mediated and, consequently, behavioral activation might be associated with Y2-receptors. Since the long C-terminal fragments, such as NPY 13–36, have never (either in brain or periphery) been attributed to antagonist properties, we hold it unlikely that NPY 13–36 in our experiments antagonized endogenous NPY to increase behavioral activity.

In a separate study, we examined whether NPY 13–36 shares the ability of NPY to

TABLE 3. Effects of NPY, PYY, NPY 13–36, PYY 13–36, and Desamido-NPY (NPY Free Acid) at Sympathetic Neuroeffector Junctions

	Direct Postjunctional Effect (Vasoconstriction)	Postjunctional Potentiation of NE-Evoked Vasoconstriction	Prejunctional Suppression of Transmitter Release
pNPY	+	+	+
pPYY	+	+	+
pNPY 13–36	o	o	+
pPYY 13–36	o	o	+
Desamido-hNPY	o	o	o

+ indicates activity.
o indicates no or very low activity.

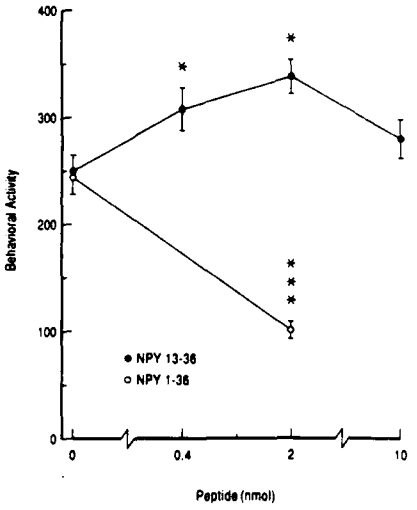

FIGURE 2. Opposite effects of intracerebroventricularly injected NPY 1–36 and NPY 13–36 on behavioral activity in the rat. Activity was assessed by open field testing and units are horizontal activity (number of line crossings during 15 min; total area 100 × 100 cm divided by lines into 25 equally sized squares). Error bars represent SEM. *Indicates that the group mean differs from that of controls on Duncan's multiple range test at $\alpha = 0.05$. See text and REFERENCE 46 for further information.

widely affect steady-state brain concentrations of NE and dopamine (DA) as well as their major respective metabolites, MHPG and DOPAC.[47] The outcome of this study showed regional differences in the effects of NPY 13–36, possibly indicating that both Y1- and Y2-receptors have the capacity to increase NE and DA turnover, and thus probably their utilization. Of the two catecholamines, DA was relatively more affected, as compared to NE by NPY, and (in some cases) by NPY 13–36.[47]

Many investigators have been concerned with the potent effects of NPY on ingestive (eating) behavior (cf. REFS. 4,5,11). Several lines of evidence indicate that the Y1-receptor is responsible for this effect of NPY. Thus, in our experiments on behavioral activity, an *en passent* finding was that NPY 13–36 did not appear to stimulate feeding to the same extent as NPY 1–36.[45] However, the possibility that C-terminal NPY fragments might increase food intake was studied more thoroughly by using NPY 2–36, NPY 9–36, NPY 14–36, NPY 21–36 (REF. 48), and NPY 20–36 and NPY 26–36 (REF. 49). In the two latter studies it was clearly shown that removal of the C-terminal markedly reduced feeding activity, in agreement with an action of NPY via Y1-receptors. Interestingly, in this volume evidence is presented that NPY 2–36 is more potent than NPY 1–36 in stimulating food intake.

In vitro, primarily by the work of Schwartz and colleagues, several cell lines have been shown to possess an exclusive population of Y1-, and no Y2- receptors (for *vice versa*, see below). Thus, several types of human neuroblastoma cells, *e.g.*, SK-N-MC, which was used to generate the data shown in TABLE 4, were found to selectively bind Y1-receptor ligands, *i.e.*, NPY, PYY and [Pro^{34}]NPY analogs (cf. REFS. 19, 43, and 44, and TABLE 4). The rat pheochromocytoma cell line, PC-12, seems to also harbor the Y1-receptor only.[44]

Y2-Receptors

Consistent with our initial observations that stimulation of the Y2-receptor is often associated with inhibition of transmitter release[4,33–35] (TABLE 3), several researchers have found that long C-terminal fragments can mimic the effect of NPY (or PYY) on

transmitter release and on parameters that reflect transmitter release. Thus, in the periphery, long C-terminal fragments, such as NPY 13–36, seem to inhibit release from sympathetic,[5,33–35] parasympathetic,[45] and sensory nerves.[50,51] Moreover, it appears that hippocampal NPY receptors are associated with inhibition of (glutamate) release[52] and in combination with binding data[18,19,44] it is reasonable to assume that presynaptic hippocampal NPY receptors are of the Y2-subtype. This assumption is supported further by the observation that NPY 20–36 like NPY 1–36 enhanced memory retention, possibly by acting on the Y2-receptors of the rostral portion of the hippocampus.[49]

As mentioned above, there seem to exist central NE and DA terminals equipped with Y2-receptors,[47] possibly related to behavioral activation.[46]

Another example of a probable Y2-receptor is the rat small intestinal receptor referred to as "PYY-preferring" by Servin *et al.*[27] These authors found that fragments as short as PYY 22–36 exhibited substantial activity in inhibiting stimulated cAMP production and competing with ^{125}I-PYY binding in these intestinal membranes.[27] As with Y1-receptors, there are several human neuroblastoma cell lines that harbor Y2-receptors only.[19,43,44] An example of such a cell line is SK-N-BE2 (kindly provided by Dr. R. Ross, Fordham University) and binding data (intact cells) consistent with the presence of the Y2-receptors are shown in TABLE 4.

The past year has seen two attempts to design centrally truncated NPY analogs with retained receptor activity.[53,54] These analogs, having preserved C-termini, can be assumed to bind well to Y2-receptors, and indeed they potently displace ^{125}I-NPY binding to brain membranes;[53,54] one analog, [D-Cys^{7},Aoc$^{8-17}$,Cys^{20}]pNPY, developed by Krstenansky *et al.*,[53] is particularly impressive in this respect. Interestingly, NPY 1–4-Aca-25–36, developed by Beck *et al.*,[54] retained substantial activity in the blood pressure test, possibly indicating that this analog has affinity to Y1-receptors as well as to Y2-receptors.

Other Possible Criteria for Subclassifying Y1- and Y2-Receptors

Although the use of fragments and analogs has proven a useful and reliable means of distinguishing between Y1- and Y2-receptors, it is still possible that additional knowledge

TABLE 4. Competition Binding to Human Neuroblastoma Cells[a]

	Y1 (SK-N-MC)	Y2 (SK-N-BE2)
pNPY	2.1 ± 0.18	0.87 ± 0.15
pPYY	0.86 ± 0.12	0.49 ± 0.11
p[Ile^{31},Pro^{34}]NPY	1.7 ± 0.21	430 ± 170
pNPY13–36	880 ± 260	5.4 ± 0.40
pNPY22–36	> 1000	9.7 ± 0.33
Desamido-hNPY	> 1000	> 1000
hPP	> 1000	> 1000

[a]Values are IC_{50} ± SEM (nM), *i.e.*, peptide concentrations required for the displacement of 50% of ^{125}I-PYY (New England Nuclear). Binding studies were performed on intact SK-N-MC or SK-N-BE2 cells in 6-well culture plates with approximately 10^{6} cells per well. Approximately 10^{-10} M radioligand was incubated with or without competing peptides for 45 min at 37°C. Monoiodinated ^{125}I-PYY was chosen as radioligand because it has very low nonspecific binding and labels both high- and low-affinity sites.[10,16] The present data were analyzed assuming a one-site model fit. Experiments were performed in triplicate (n = 3–6). It is concluded that SK-N-MC represents a Y1- receptor-containing cell line while SK-N-BE2 has a population of Y2-receptors; see text for further information.

about NPY (and PYY) receptors might arise as a result of attempts to subclassify phenomena that could be selectively associated with Y1- but not Y2-receptors or *vice versa*. A few possibilities will be entertained in this article: a) Are different second messengers associated with the different receptors? b) Are the receptor subtypes differentially sensitive to pertussis toxin? c) Do the relative potencies of NPY and PYY for Y1- and Y2-receptors differ? d) Are (some of) the Y1-receptors functionally coupled to α_2-adrenoceptors?

a) Are different second messengers associated with the different receptors? This possibility was raised by us a few years ago.[5,35] It is well accepted that most, if not all, NPY receptors may couple to inhibition of adenylate cyclase and hence decreased levels of cAMP (reviewed, *e.g.*, in REFS. 5 and 11). As shown in TABLE 5, NPY decrease forskolin-stimulated cAMP levels in SK-N-MC (Y1-receptor) as well as in SK-N-BE2 cells (Y2-receptor). In agreement with the binding studies shown in TABLE 4, [Ile^{31},Pro^{34}]NPY and NPY13–36 selectively affected cAMP accumulation in SK-N-MC and SK-N-BE2, respectively (TABLE 5). On the other hand, PC-12 cells, which in binding studies appear to have Y1- receptors (*e.g.*, REF. 44) may not couple to inhibition of adenylate cyclase.[55] Hence, measurement of cAMP will probably not be a means to distinguish Y1- from Y2- receptors.

TABLE 5. Inhibition of Forskolin-Stimulated cAMP Concentrations in Neuroblastoma Cells[a]

	Y1 (SK-N-MC)	Y2 (SK-N-BE2)
pNPY	22 ± 8.1	7.8 ± 3.5
p[Ile^{31},Pro^{34}]NPY	9.6 ± 4.0	> 1000
pNPY13–36	> 1000	36 ± 11

[a]Values are IC_{50} ± SEM (nM). Neuroblastoma cells (approximately 10^6 per well) were grown and preincubated like cells used for binding studies on intact cells (TABLE 4). The peptides were added 15 min before 3 μM forskolin, which was used to elevate cAMP concentrations. Cells were then incubated for 10 min and cAMP was subsequently measured by radioimmunoassay.[97] The E_{max} of all three peptides were similar and corresponded to a reduction of cAMP concentrations from 150–220 to 70–105 pmol (SK-N-MC) and 80–110 to 35–60 pmol (SK-N-BE2) per 10^6 cells. The experiment was performed in triplicate; n = 3–4.

In some cell types, dorsal root ganglion cells,[51] human erythroleukemia cells,[56] and SK-N-MC cells,[57] NPY has been shown to mobilize intracellular calcium ions,[51,56,57] and, in addition, NPY had rather weak direct or potentiating effects on phosphatidyl inositide (PI) turnover in brain,[5,35,58] vas deferens,[59] and vasculature.[21] At the present time, it cannot be excluded that NPY's effect on PI turnover is secondary to the more powerful effect on intracellular calcium ion mobilization which in turn may get to stimulate phospholipase C resulting in an apparent PI response.[21] Interestingly, it has been suggested that NPY (acting on Y1-receptors) may be targeting a discrete, thapsigargin-sensitive pool in the endoplasmic reticulum that does not take part in the signalling of calcium ion entry.[57] In any event, it appears clear that the Y1-receptor is capable of mobilizing intracellular calcium ions in some cells,[57] but it remains to be established whether the Y2-receptor has the same capacity. The dorsal root ganglion cells studied by Miller's group clearly have an NPY-calcium ion coupling, and these cells could indeed be equipped with Y2-receptors since NPY 13–36 did evoke PI hydrolysis, however less pronounced than NPY1–36.[51]

In conclusion, we feel that measurement of second messengers, in the belief that they

may be differentially associated with Y1- and Y2-receptors, does *not* provide a basis for subclassification. The differences that have been reported are more likely to be due to differences between cell types than between NPY (PYY) receptor coupling. Nevertheless, measurement of second messengers may be very useful when screening of heterologously expressed NPY receptors (see section below).

b) Are the receptor subtypes differentially sensitive to pertussis toxin? A number of reports on various cell types have claimed that pertussis toxin abolishes or greatly attenuates NPY responsiveness.[51,56,60–67] However, a few other studies have resulted in limited[5] or no effects at all[68,69] of the toxin. Thus, in hippocampus[68] and sympathetic nerve terminals in heart,[69] which are probably Y2-receptor-containing tissues (cf. above), pertussis toxin was without effect. On the other hand, effects that are probably Y1-receptor-mediated seem highly sensitive to the toxin.[56,60,63–67] The thorough studies by Miller and colleagues on cultures of rat sensory neurons clearly showed that pertussis toxin abolishes the ability of NPY (and other neurotransmitters) to inhibit calcium ion currents and that the effects of the toxin can be overcome by introduction of the α subunit of the G-protein, Go, into the neurons.[51,61,62] It appears that the proposed concept of selective pertussis toxin sensitivity of the Y1-receptor would infer that the latter authors indeed were studying this subtype. However, since they found NPY13–36 to be active, although less so, in their system,[51] it is possible that their data imply that the Y2-receptor can also be affected by pertussis toxin.

In conclusion, many effects of NPY can be blocked by pretreatment with pertussis toxin. At the present time, it cannot be excluded that the Y2-receptor is less sensitive to the toxin as compared to the Y1-receptor.

c) Do the relative potencies of NPY and PYY for Y1- and Y2-receptors differ? This possibility is raised as a consequence of results obtained by many research groups, indicating that the relative potencies of NPY and PYY differ between various experimental systems. In one instance, the effect of NPY was even found to be opposite that of PYY.[69] In our experience, the Y2-receptor is significantly more sensitive to PYY than NPY (*e.g.*, REFS. 5,33 and TABLE 4). On the other hand, the peptides are almost equipotent at the Y1-receptor (REFS. 5, 33 and TABLE 4). Going through the literature, this concept (NPY ≈ PYY at Y1- and NPY < PYY at Y2-receptors is essentially supported, however several inconsistencies occur. Obviously, these inconsistencies may be attributed to use of different batches of peptides from many different suppliers. Therefore, it may not be completely relevant to compare data from various laboratories, though if data are consistent in the work of a given laboratory, it may be possible to subclassify receptors by use of NPY and PYY only. This approach was successfully employed by Lynch *et al.*,[10] who were able to selectively label different classes of receptors by using ^{125}I-labelled NPY and PYY. Among areas these authors found to be preferentially labeled by ^{125}I-PYY is hippocampus, and this finding appears consistent with the fragment data indicating that hippocampal NPY receptors are primarily of the Y2-subtype (see above). An alternative interpretation of their data could be that ^{125}I-PYY labelled both high and low affinity sites while ^{125}I-NPY labelled low affinity sites only.[10]

In conclusion, it cannot be excluded that use of NPY and PYY may enable differentiation between Y1- and Y2-receptors. However, the use of fragments and analogs in accordance with previously mentioned guidelines will clearly offer a more advantageous route.

d) Are (some of) the Y1-receptors functionally coupled to α_2-adrenoceptors? Many studies have resulted in clear-cut interactions between NPY-receptor- and α-adrenoceptor mechanisms.[46,62,66,71–75] More specifically, it is likely that, at least in brain, the α_2-adrenoceptor is related to NPY-ergic transmission and possibly the Y1-receptor. Thus, in brain α_2-adrenergic antagonism can attenuate NPY-evoked blood pressure responses,[63,74,75] sedation/anxiolysis,[46] inhibition of NE release,[66] feeding,[71] and potas-

sium conductance in cells of locus coeruleus.[72] In pancreatic islets, phentolamine (a nonspecific α-antagonist) reversed glucose-induced insulin secretion,[73] while in kidney cortical collecting tubule either α_2-adrenergic receptor blockade (yohimbine) or occupancy (clonidine) prevented NPY-induced inhibition of arginine vasopressin action.[63] Fuxe and coworkers have investigated the possibility that α_2-adrenergic drugs and NPY interact reciprocally at their respective binding sites.[74,75] Several of their studies have indicated intricate receptor-receptor interactions (*e.g.*, REFS. 74,75). However, it is difficult to evaluate if the degree of interactions in these binding studies matches that observed in functional studies. In our experience, in studies of cell lines with exclusive populations of Y1- or Y2-receptors (cf. TABLE 4) or brain homogenates, adrenergic drugs have no or very limited effects on binding characteristics of ^{125}I-NPY or ^{125}I-PYY. Therefore, we hold it unlikely that NPY/PYY and adrenergic drugs share the same binding site. Rather, binding interactions would seem to be secondary to effects on a common signal-transduction system not shared by, *e.g.*, opioid μ-receptors.[72] (In this context, it may be noted that it is highly unlikely that vasoconstrictor synergism of NPY and α_1-adrenergic agonists reflects an interaction at their respective recognition site, REF. 21.) Finally, the mechanism by which NPY and α_2-adrenergic drugs interact is probably sensitive to pertussis toxin.[63–66] Hence, it is possible that, at least in some cell types, the Y1-receptor (as defined previously) may be mediating the α-blockade sensitive actions of NPY.

Short NPY Fragments (and NPY Itself) Can Induce Hypotension

A few years ago we noted that C-terminal NPY fragments in principle have the capacity to lower systemic blood pressure, *i.e.*, to induce the opposite effect of the parent molecule.[5] This phenomenon has also been studied in detail by Brown and colleagues, who have concentrated their efforts on NPY 18–36, which, in high doses, however dose-dependently, reduces blood pressure.[76,77] It was argued that the latter fragment does not act by antagonizing endogenous NPY.[76]

Recently, we attempted to further characterize this phenomenon.[36,37] We then found NPY itself to cause a biphasic blood pressure response in conscious[37] and anesthetized intact[36] and areflexive pithed[36,37] rats. The threshold dose of NPY given i.v. as a bolus for the biphasic response, is higher than the dose required for evoking the well-known vasopressor response only.[36,37] In addition, we have noted that equally high doses of NPY 18–36, NPY 20–36 and NPY 22–36 (10–300 nmol/kg) are able to elicit a biphasic response in both conscious and pithed rats,[37] however the pressor component being much less potently elicited by the fragments. In contrast to the protracted "parabola-like" pressor effect of NPY, the fragments generally elicited a very short-lasting "spike-like" rise in pressure (FIG. 3). However, the fragments were relatively more effective than NPY with regard to inducing a vasodepressor response lasting several minutes (FIG. 3). The order of vasodepressor potency among the three fragments was NPY 18–36 > NPY 22–36 > NPY 20–36 (NPY 26–36 was much less active).[37] We have usually found this drop in blood pressure to be accompanied by a rise in heart rate. Because this is seen also in the pithed rats, which are areflexive, it is probably not entirely due to reflex tachycardia.[37]

Thus, both NPY and C-terminal NPY fragments are associated with a decrease in systemic blood pressure. Since the (short) fragments largely lack vasopressor activity, they appear to be more selective depressors. This "shift" from largely pressor to largely depressor is gradual and the most promising (natural) fragments are NPY 18–36[37,76,77] and NPY 22–36.[37] However, it must be noted that the potency of these fragments (as well as of NPY) in eliciting depressor responses is low, which obviously raises the question if

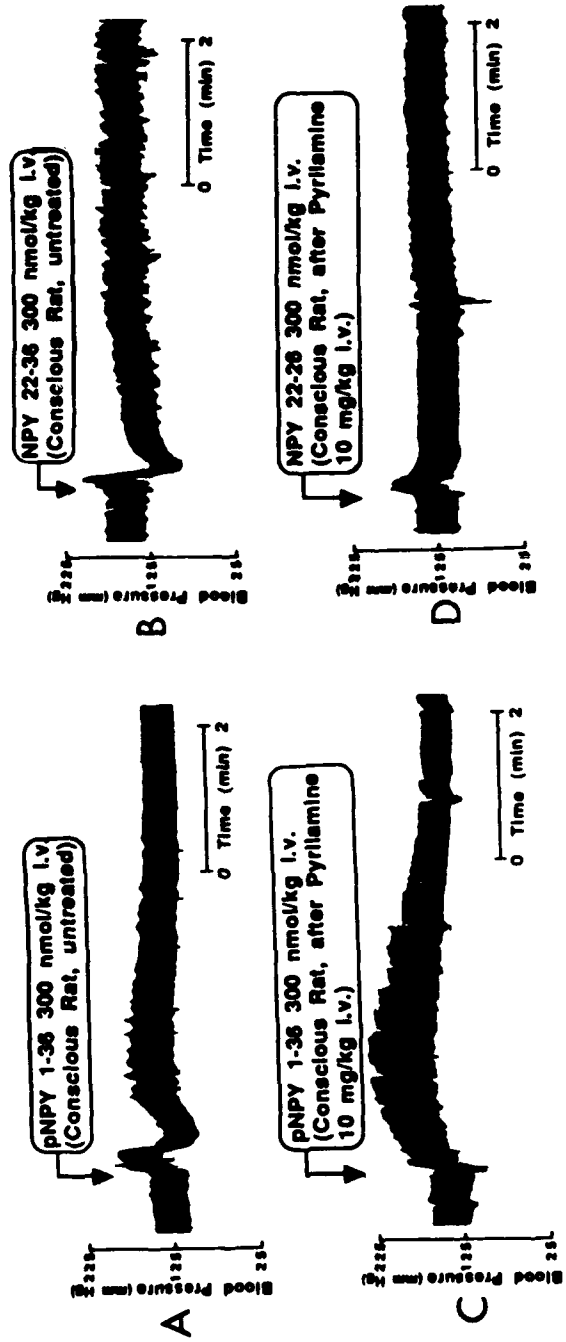

FIGURE 3. Tracings of blood pressure recordings in conscious rats. Biphasic response to pNPY 1–36 (300 nmol/kg; *panel A*) and NPY 22–36 (300 nmol/kg; *panel B*). Note that the fragment evokes a pressor response of shorter duration and a more pronounced depressor response. Pyrilamine, a histamine-H1-antagonist, at 10 mg/kg, blocks the depressor response to pNPY 1–36 (*panel C*) as well as to NPY 22–36 (*panel D*), revealing a sustained ("parabola-like") pressor response to pNPY 1–36 (*C*) but not to NPY 22–36 (*D*). It is concluded that the fragment has less vasopressor activity and it is therefore a relatively more selective vasodepressor agent. Both peptides may release histamine from mast cells to reduce blood pressure. See text for further discussion.

the effect is physiologically significant. Nevertheless, these experiments[36,37,76,77] sought to investigate systemic effects and it is still possible that the phenomenon may be of importance in selected local blood flow.

In these experiments (FIG. 3 and REFS. 36 and 37) showing slowly emerging and long-lasting vasodepressor activity of rather large doses of NPY, it cannot be excluded that shorter fragments were generated upon NPY hydrolysis. If so, such (C-terminal) fragments might be Y2-selective agonists capable of inhibiting sympathetic outflow.

We have possibly gained some insight into the mechanism(s) by which the fragments lower blood pressure. As expected based on studies *in vitro* they did not antagonize the vasopressor effects of NPY (or NE).[37,76] While it is conceivable that they, as opposed to NPY 1–36, selectively activate Y2-receptors to inhibit stimulated (or tonic) sympathetic transmitter release (as described in some detail above), and thereby contribute to the lowering of blood pressure, we would also like to propose an additional intermediary link. We found that the histamine-H1-antagonist, mepyramine, or the histamine liberator com-

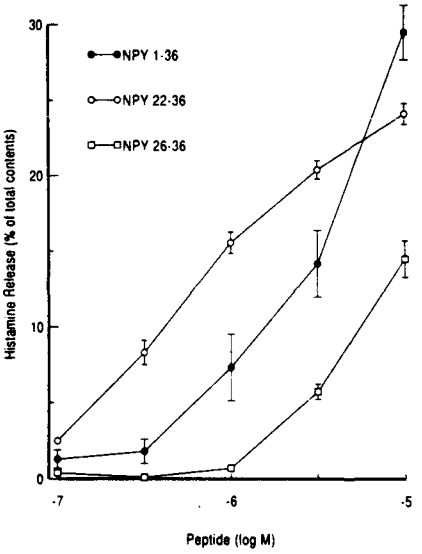

FIGURE 4. Histamine release from peritoneal mast cells evoked by pNPY 1–36, NPY 22–36 and NPY 26–36. Mast cells were prepared and studied as described previously,[98] and histamine was measured by a fluorometric assay.[99] Values are means of 3–4 separate determinations and error bars represent SEM.

pound, 48/80, abolished or markedly attenuated *depressor* responses to NPY as well as to the fragments.[36,37] These findings suggested that mast cells might release histamine when stimulated by NPY or NPY fragments. Indeed, the peptides *in vitro* released histamine from peritoneal mast cells (REF. 37 and FIG. 4). Interestingly, NPY 22–36 was at least as potent as NPY 1–36. Since, in addition, NPY 26–36 retained some activity on mast cells, while being inactive in Y2-receptor assays (*e.g.*, the electrically paced vas deferens[78]), the mast cells thus appear to possess atypical NPY receptors, distinct from previously defined Y1- and Y2-receptors.

Can Yet Other Messengers Serve as Mediators of NPY's Effects?

Thus, mast cells and their histamine are probably mediators of some vascular actions of NPY. There is also evidence of other indirect NPY effects. For instance, protanoids

have been implicated (*e.g.*, REFS. 79–81). In rat small intestine, where NPY and PYY are antisecretory, endogenous prostanoid production appears crucial since the effects cannot be demonstrated in the presence of the cyclooxygenase inhibitor, piroxicam.[80] Moreover, some vasoconstrictor actions of NPY may depend on prostanoids. Hence, the powerful coronary constriction is attenuated by either cyclooxygenase blockade or thromboxane receptor blockade and, in addition, intracoronary NPY releases thromboxane.[81]

Another possible mediator of vascular NPY actions is the endothelial cell, however probably only in selected vascular beds. Thus, the integrity of the endothelium was crucial for maintaining the potentiating effect of NPY on NE-evoked vasoconstriction in rabbit ear artery[82] and rat superior mesenteric artery[83] but not in rabbit pulmonary artery.[21]

Are NPY/PYY Fragments Generated In Vivo?

A frequently arising question addressed to investigators utilizing NPY/PYY fragments (to delineate structure-activity relationships and to selectively affect putative receptor subtypes) is whether the fragments occur endogenously. In other words, are the fragments, which undoubtedly constitute useful pharmacological tools, also mimicking physiologically relevant events? There is unfortunately very little data to answer this question. Nevertheless, PYY 3–36 seems to exist in both human[84] and canine[85] colonic mucosa; it can be assumed that this naturally-occuring fragment has greater affinity to Y2- than to Y1-receptors (cf. text above). Moreover, NPY 1–30 has been detected in both bovine adrenal glands and brain, but it is not clear if this implies the existence of NPY 31–36.[86]

Finally, when analyzing human cerebrospinal fluid by HPLC, we have observed several NPY-immunoreactive peaks perhaps corresponding to hydrolyzed fragments.[87] Since, in contrast, brain tissue largely contained authentic NPY, it is possible that NPY fragments are generated upon release to CSF.[87]

Does NPY (PYY) Interfere with Sigma and Phencyclidine Binding?

Very recently, it was reported that NPY and PYY interact with rat brain sigma and phencyclidine (PCP) binding sites.[88] While being essentially equipotent at sigma sites, PYY was more potent than NPY at PCP sites.[88] This possibility is intriguing in view of proposals of the existence of endogenous peptide ligands for these sites.[89,90]

Heterologous Expression of NPY/PYY Receptors in Xenopus *Oocytes*

The *Xenopus laevis* oocyte has been extensively used in biological research both for developmental studies and for efficient mRNA translation and posttranslational processing of receptors and ion channels (*e.g.*, REFS. 91–94). Of particular interest in the present context is the potential of the oocyte to serve as an expression cloning system for receptors as pioneered by Nakanishi and colleagues.[92] In various attempts to expression clone NPY/PYY receptor(s), we have pursued detection of *de novo* expressed NPY/PYY receptor(s) by the following methods: (1) electrophysiology; (2) intracellular calcium ion imaging; and (3) cAMP measurement.

Electrophysiology

In the oocyte, stimulation of a number of endogenous (*e.g.*, muscarinic) and *de novo* expressed receptors results in PI hydrolysis and inositol triphosphate-evoked intracellular

release of calcium ions. The calcium ions in turn activate chloride currents which are easily detected in a voltage clamped oocyte.[91-94] These powerful depolarizing currents have allowed the expression cloning of the substance K[92] and serotonin 1C[93] receptor. In these cases mRNAs were initially extracted from stomach and choroid plexus, respectively, and when currents were detected in the oocytes, expression libraries were constructed, and following fractionation single receptor clones were eventually obtained.[92,93]

However, based on our experience it seems unlikely that either the Y1- or the Y2-receptor couples well to PI hydrolysis when expressed in the oocytes. For instance, while we have found various brain mRNA preparations to be faithfully translated into functional receptors for other neurotransmitters/neuropeptides (*e.g.*, glutamate, substance P, cholecystokinin, thyrotropin-releasing hormone, and neurotensin), application of NPY has

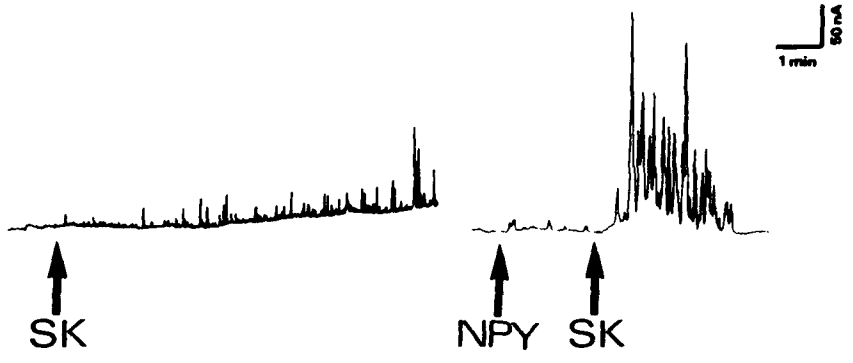

FIGURE 5. Tracings of electrophysiological recording of a *Xenopus* oocyte, voltage clamped at −60 mV. Three days prior to this experiment the oocyte had been microinjected with 30 ng mRNA extracted by standard guanidinium isothiocyanate/oligo(dT)cellulose methods from bovine lung and 1 ng *in vitro* transcribed mRNA corresponding to the cloned bovine substance K-receptor (SKR).[92,94] The latter *de novo* expressed receptor renders the oocyte sensitive to substance K in a concentration-dependent manner.[92,94] At a threshold concentration of substance K, approximately 5 nM, minor (chloride) currents are observed *(left)*. However, when pNPY (100 nM) was applied prior to substance K, the latter peptide evoked much larger currents *(right)*. This phenomenon has been observed in over 20 oocytes. In oocytes injected with substance K receptor mRNA only, threshold enhancement phenomena associated with NPY pretreatment have never been observed (not shown). It is concluded that lung mRNA is expressed to confer to the oocyte NPY's ability to enhance the response to substance K.

generally resulted in no or minor currents. Therefore, we hold it unlikely that electrophysiological detection alone will prove useful when screening oocytes for expression of NPY/PYY receptors represented in cDNA libraries.

Interestingly, however, we have evidence that NPY, by stimulating heterologous NPY/PYY receptors, has the capacity to help PI-coupled agonists overcome response thresholds for evoking chloride currents, *i.e.*, NPY can potentiate responses to (PI-coupled) agonists; this situation is analogous to potentiation of vasoconstriction (see above and REF. 21). The phenomenon was observed after coexpression in oocytes of mRNA from lung (a tissue which probably contains both Y1- and Y2- receptors based on physiological studies referred to above) and *in vitro* transcribed mRNA for the cloned substance K receptor.[92,94] When stimulating the latter *de novo* expressed receptor with threshold concentrations of the agonist, substance K (cf. REF. 94), we observed that

preexposure to NPY (100 nM) enhanced substance K-evoked chloride currents (FIG. 5). Thus, we conclude that lung mRNA was expressed in the oocytes and thereby conferred the ability of NPY to potentiate the response to substance K.

Intracellular Calcium Ion Imaging

As pointed out, NPY has been associated with intracellular calcium ion mobilization in several cell types.[51,56,57] Therefore, we have addressed the question as to whether *de novo* expressed NPY/PYY receptors in *Xenopus* oocytes couple to the release of calcium ions, perhaps independent of PI hydrolysis. By imaging oocytes with a digital imaging fluorescence microscopy system using the calcium sensitive dyes fura-2 and fluo-3 we have recently argued that several endogenous and *de novo* expressed neurotransmitter/hormone receptors are linked to mobilization of intracellular calcium ions.[94] Preliminary evidence using fluo-3 has indicated that expression of lung mRNA confers NPY responsiveness (six of eight oocytes; no response in any of ten vehicle-injected oocytes). The latter finding appears compatible with a view that stimulation of NPY/PYY receptors, by raising intracellular calcium ion concentration (with kinetics different from that associated with PI hydrolysis), may facilitate PI coupled events such as contraction of smooth muscle[21] and chloride currents in oocytes (see previous paragraph and FIG. 5).

cAMP Measurement

Individual *Xenopus* oocytes can be assayed for cAMP, however the basal adenylate cyclase activity is low.[95,96] Therefore, when screening oocytes for expression of a receptor that, like the Y1- as well as the Y2-receptor, can be assumed to be associated with reductions in adenylate cyclase activity and cAMP concentrations (cf. TABLE 5), one has to find a means for raising the latter. However, in our hands and others,[96] forskolin, which activates adenylate cyclase in a receptor-independent fashion in a variety of systems (*e.g.*, the neuroblastoma cells of TABLE 5), causes only a moderate increase in cAMP levels. Thus, other methods are necessary, such as coexpression of receptors that stimulate cAMP levels. Recently, this was successfully accomplished by using *in vitro* transcribed β_2-adrenergic receptor mRNA;[96] this receptor is not endogenous to the oocyte proper.[95] Accordingly, in our laboratory we have coexpressed the β_2-adrenergic receptor (5–6 ng mRNA oocyte; the cDNA was generously provided by Dr. Catharine D. Strader, MSD, Rahway, MJ) and mRNA isolated from the Y1-receptor-containing SK-N-MC neuroblastoma cells. As shown in FIGURE 6, NPY (up to 1 μM) was inactive *per se* but inhibited isoprotenenol-induced elevation of cAMP concentrations. Thus, it is likely that the Y1-receptor, when expressed in *Xenopus* oocytes, like in its native environment, has the capacity to inhibit adenylate cyclase activity.

In conclusion, many receptors have proven much easier to detect upon *de novo* expression in *Xenopus* oocytes than NPY/PYY receptors. This is primarily due to the apparent inability of the latter to couple well to PI hydrolysis. Nevertheless, we have delineated a few strategies to detect functional expression (coexpression of other receptors followed by electrophysiology or cAMP measurement, and possibly by calcium ion imaging). As a complement to eucaryotic expression, the *Xenopus* oocyte may be a useful heterologous expression cloning system for NPY/PYY receptors. Our data indicate that these heterologous receptors have similar characteristics as in their native environment.

SUMMARY

Heterogeneity among NPY (and PYY) receptors was first proposed on the basis of studies on sympathetic neuroeffector junctions, where NPY (and PYY) can exert three

types of action: 1) a direct (*e.g.*, vasoconstrictor) response; 2) a postjunctional potentiating effect on NE-evoked vasoconstriction; and 3) a prejunctional suppression of stimulated NE release; the two latter phenomena are probably reciprocal, since NE affect NPY mechanisms similarly. It was found that amidated C-terminal NPY (or PYY) fragments, *e.g.*, NPY 13–36, could stimulate selectively prejunctional NPY/PYY receptors, which were termed Y2-receptors. Consequently, the postjunctional receptors which were activated poorly by NPY/PYY fragments, were termed Y1-receptors. Later work has indicated that the Y2-receptor may occur postjunctionally in selected sympathetic effector systems. The central nervous system appears to contain a mixture of Y1- and Y2-receptors as indicated by functional as well as binding studies. For instance, NPY and NPY 13–36 produced diametrically opposite effects on behavioral activity, indicating the action of the parent peptide on two distinct receptors.

Cell lines, most importantly neuroblastomas, with exclusive populations of Y1- *or* Y2-receptors, have been characterized by binding and second messenger studies. In this work, selective agonists for the two receptor subtypes were used.

Work of many investigators has formed the basis for subclassifying NPY/PYY effects being mediated by either Y1- or Y2-receptors. A preliminary subclassification based on effects of NPY, PYY, fragments and/or analogs is provided in TABLE 6. It is, however, to be expected that further receptor heterogeneity will be revealed in the future.

It is argued that mast cells possess atypical NPY/PYY receptors. The histamine release associated with stimulation of the latter receptors may, at least in part, underlie the capacity of NPY as well as of short C-terminal fragments to reduce blood pressure. Fragments, such as NPY 22–36, appear to be relatively selective vasodepressor agents because of their weak vasopressor properties.

Finally, as a result of attempts to expression clone NPY/PYY receptor(s), we have, by use of *Xenopus* oocytes, designed a few strategies to detect *de novo* NPY/PYY receptor expression. Thus, it is suggested that mRNA derived from lung and neuroblastoma cell lines may confer NPY responsiveness to the oocytes as assessed by electrophysiological and second messenger studies.

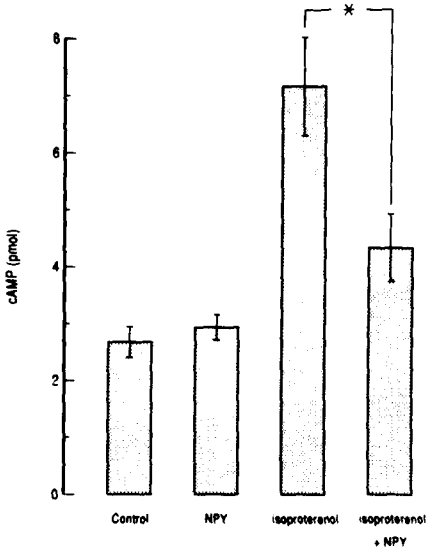

FIGURE 6. Functional expression of NPY receptors in *Xenopus* oocytes assessed by the ability of NPY to reduce cAMP concentrations. Three days prior to cAMP measurement of individual oocytes, these had been microinjected with vehicle (control) or a combination of 30 ng mRNA from Y1-receptor-containing SK-N-MC cells (see text) extracted by standard guanidinium isothiocyanate/oligo(dT)cellulose methods *and* 5 ng of *in vitro* transcribed β_2-adrenoceptor mRNA. The co-injected oocytes were incubated for 45 min in the presence of either pNPY (100 nM), isoproterenol (100 μM) or both compounds. It was found that isoproterenol, but not pNPY, stimulated the accumulation of cAMP, measured by radioimmunoassay following acetylation.[97] However, the data indicate functional expression of NPY receptors since the peptide significantly reduced ($p < 0.05$) isoproterenol-induced cAMP accumulation. Results are means ± SEM of determinations on 8–10 oocytes from the same toad donor. See text for further information.

TABLE 6. Effects Possibly Associated with:[a]

	Reference	Comment
Y1-receptors		
Vascular smooth muscle contraction	5,33–35,43,45	major vascular receptor
Sedation/anxiolysis	46	
Centrally evoked hypotension	Fuxe *et al.*, this volume	
Food intake	48,49; several papers, this volume	NPY 2–36 very potent
Increased transmitter turnover	47	less so than Y2
Reduced cAMP accumulation	57; this paper	like Y2
Calcium ion mobilization	57; this paper	also when *de novo* expressed
Potentiation of PI-coupled agonists	5,33–35; this paper	also when *de novo* expressed
Y2-receptors		
Inhibition of transmitter release	5,33–35,45,50–52	variety of nerves
Vascular smooth muscle contraction	5,41,45; this volume	selected vasculature
Increased transmitter turnover	47	
Behavioral activation	46	
Centrally evoked by hypertension	Fuxe *et al.*, this volume	
Enhanced memory retention	49	
Intestinal secretion	27; Cox *et al.*, this volume	
Platelet aggregation	Myers *et al.*, this volume	
Reduced cAMP accumulation	this paper	

[a]Please see text for further explanation.

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Biologically Active Neuropeptide Y Analogs[a]

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INTRODUCTION

The synthesis and biological characterization of neuropeptide Y (NPY) analogs with the aim of understanding relationships between its structure and activity has been ongoing in our laboratories since 1987. Upon definition of a bioactivity that would serve as a basis for structure-activity studies we followed a general iterative approach based on design, synthesis and testing of analogs. The bioactivity we chose to use in the present studies was the measurement of cardiovascular parameters in conscious rats upon intraarterial (i.a.) administration of the analogs. In particular we were interested in elucidating the mechanism by which NPY elicits a short-lived hypertension upon i.a. administration. We then sought to define the minimum structure analog for that bioactivity by systematic deletions of either terminus of the native structure. The contribution of single residues to the bioactivity was probed by the synthesis of analogs with systematic D-substitutions. It was then possible to integrate these findings and resolve the structure of the NPY molecule into putative pharmacophores. The design-synthesis-testing cycle was repeated following the observation that the NPY fragment NPY_{18-36} elicited hypotension upon i.a. administration. We report here the results of these studies in which we have synthesized and tested in the conscious rat bioassay NPY analogs of the following classes: 1. NPY_{1-36} analogs with N- and C-terminal modifications and D-substitutions in the N-and C-terminal regions; 2. Fragments of NPY_{1-36} truncated at either or both the C- and N-terminii; and 3. Fragments and analogs of NPY_{18-36}.

METHODS

Peptide Synthesis

NPY and NPY_{18-36} analogs were synthesized by methods detailed elsewhere.[1] Briefly, peptides were manually synthesized using α-butyloxycarbonyl (α-BOC) amino

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protection on methylbenzhydrylamine (MBHA) resins prepared in house by the method of Rivier *et al.*[2] or protected amino acid-chloromethyl (CM) resins prepared by the method of Horiki *et al.*[3] α-BOC-amino acids (Bachem, Torrance, CA) were coupled via dicyclohexylcarbodiimide in CH_2Cl_2 and/or DMF. Asn and Gln were coupled in the presence of a 2-fold excess of 1-hydroxybenzotriazole. Sidechain protection of α-BOC amino acids was as follows: Arg(Tos), Asp(OcHx), Glu(OcHx), His(Tos), Lys(2ClZ), Ser(Bzl), Thr(Bzl), and Tyr(2BrZ). Deblocking was accomplished with 50% trifuloracetic acid (TFA) in CH_2Cl_2 in the presence of 1% ethanedithiol for 25 min. The protected peptide-resin was cleaved in liquid hydrogen fluoride in the presence of 3% anisole at 0°C for 45 min for CM resins and 60 min for MBHA resins. The crude peptides were precipitated with anhydrous diethyl ether and separated from ether-soluble nonpeptide material by filtration. The peptides were extracted from the resin with water, and the aqueous solutions were then lyophilized.

Peptide Purification

Crude peptides were purified by preparative reversed phase–high performance liquid chromatography (RP-HPLC) usually in two steps. This methodology has been described in detail previously.[1,4,5] Reagent grade TFA and triethylamine (TEA) were distilled to constant boiling point for use in the preparation of chromatographic buffers. Briefly, gradient conditions were established by analytical HPLC. Gradient conditions for preparative HPLC were inferred from the analytical results. Vydac C_{18} columns (0.46 × 25 cm, 5 μm particle size, 30 nm pore size, Separations Group, Hesperia, CA) were used and detection was at 210 nm. The preparative HPLC system used was a modified Waters 500A Prep LC. The cartridges used were hand-packed, in house, using Waters polyethylene sleeves and frits and Vydac bulk C_{18} material, 15–20 μm particle size, 30 nm pore size. The crude lyophilized peptides (0.5–1.5 g) were dissolved in water, loaded onto the C_{18} cartridges and eluted with linear triethylammonium phosphate (TEAP) pH 2.25/acetonitrile (MeCN) gradients. 50–100-ml fractions were collected and monitored by isocratic analytical HPLC. Appropriately enriched fractions (>95% pure) were pooled, diluted an reloaded onto the preparative cartridge. The peptide was eluted with a linear gradient of 0.1% TFA/MeCN. Fractions were again collected and analyzed, and those containing the purified peptide were pooled and lyophilized.

Peptide Characterization

Purified peptides were subjected to HPLC analysis in two mobile phase systems: 0.1% TFA/MeCN and TEAP pH 2.25/MeCN on a C_{18} stationary phase and were all >92% pure. Amino acid analysis of the peptides was performed following hydrolysis in 4N methanesulfonic acid at 110°C for 24 hr to confirm amino acid composition.

Conscious Rat Blood Pressure Assay

Detailed descriptions of the methods used for the biological testing of these compounds appear elsewhere.[1,6] Male Sprague-Dawley rats (240–280 g, Bantin-Kingman Laboratories, Fremont, CA) were used. All procedures performed on these animals were in accordance with the guidelines of the University of California, San Diego Committee on Investigations Involving Animal Subjects. All animals, when used in experiments were conscious and freely moving. Catheters were inserted into the femoral artery, and were

routed subcutaneously and exteriorized between the scapulae. Catheter placement was performed on the day of the experiment under pentobarbital anesthesia (40 mg/kg). There was at least a 2-hr recovery period between the catheter placement and the start of the experiment. Saline solutions of the peptides were administered intraarterially in a 100-μl bolus. Zero points were determined prior to administration of peptides by injection of saline vehicle alone. Arterial pressure and heart rate (HR) were measured with Gould-Statham P23Db pressure transducers and monitored with a Gould physiograph. A Cyborg A-D converter linked to an IBM-XT compatible computer was used for data collection. Mean arterial pressure (MAP) was calculated as:

$$[(systolic - diastolic / 3) + diastolic].$$

Cardiac output (CO) was measured with a Cardiotherm 500 computer linked to a Cardiomax-II-R interface. 100 μl of 0.9% NaCl was injected at room temperature, and duplicate measurements made at each time interval. Systemic vascular resistance (SVR) was calculated from MAP and CO as:

$$SVR\ (resistance\ units) = MAP\ (mm\ Hg) / CO\ (l/min)$$

Stroke volume (SV) was calculated as:

$$SV\ (\mu l/beat) = CO\ (l/min) / HR\ (beats/min)$$

Data Analysis

In all cases data points were calculated as Δ's by subtraction of pretreatment zero values from posttreatment values for each animal at each dose and time. These data were then grouped for each dose and time and means ± SEM were calculated. For NPY_{1-36} analogs where short-lived hypertension was the parameter under consideration full dose response curves (0.4–40 μg/kg) were fitted to the 1-min data by computer iteration (Bolt, Beranek and Newman Research Systems RS/1 Biocomputing Software, Chicago, IL) and ED_{50}'s (the effective dose required to elicit 50% of the maximal response) were calculated. Relative potencies were then calculated as ED_{50} (compound)/ED_{50} (NPY) for those compounds for which the experimentally determined maximum (max) was within 20% of that determined for NPY. For NPY_{13-36} analogs where the longer time course of action precluded extensive dose studies, data is expressed as ΔMAP at 0.4 mg/kg, a dose which results in a submaximal response with the parent compound NPY_{18-36}.

RESULTS AND DISCUSSION

In general we found the syntheses and purification of NPY and analogs to be straightforward, provided that coupling efficiencies were monitored at each step. Several "difficult" couplings were identified, notably in the sequence Arg^{25}-His^{26}-Tyr^{27}. This cautioned the use of automated synthesis without monitoring as poor coupling efficiencies at these points in the synthesis may generate bioactive fragments.

NPY_{1-36}

We showed previously[1] that NPY elicits a rapid-onset, short-lived hypertension upon intraarterial administration in the conscious rat bioassay of doses up to 40 μg/kg. The

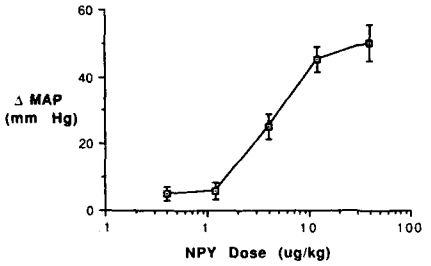

FIGURE 1. Dose response curve for NPY effect on MAP. Measurements made 1 min following injection of peptide. *Points* represent means ± SEM, n = 13.

ED_{50} for this response was 3.04 ± 0.88 μg/kg (mean ± SEM, n = 13) (FIG. 1), and a maximal dose (40 μg/kg) elicited an increase in MAP from 107.0 ± 2.6 to 157.0 ± 5.5 mm Hg (mean ± SEM, n = 13). Readministration after 10 min gave a repeated and equivalent response with no evidence of tachyphylaxis. The maximal dose slowed HR but not to a statistically significant degree. We have since explored further[6] the mechanism of this response and found that NPY elevated SVR to a maximal level within 1 min of administration. SVR remained elevated for more than 30 min. CO decreased more slowly and reached a nadir after 5 min. CO then remained depressed for more than 30 min. Accordingly, MAP rose only transiently and returned to baseline values within 5 min.

NPY_{1-36} Analogs

To define the contribution of single residues to the integrity of the hypertensive action of NPY, single D-substitutions were made throughout the molecule. FIGURE 2 gives the relative hypertensive potencies of NPY_{1-36} analogs with D-substitutions in the N-and C-terminal regions of the molecule and several N-terminally modified analogs. It is evident that D-substitutions may be made to the N-terminal region of NPY up to position

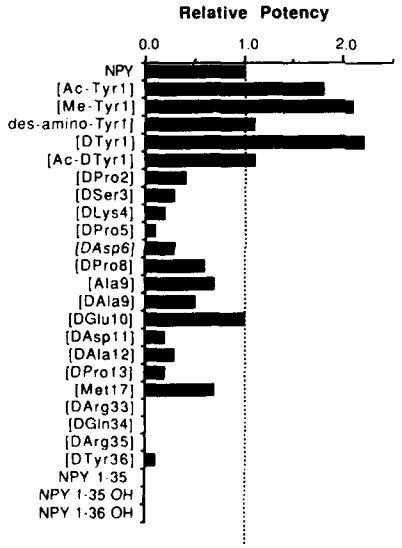

FIGURE 2. Relative potencies of NPY_{1-36} analogs calculated as ED_{50} analog/ED_{50} NPY. ED_{50}'s determined from computer fitting of dose-response curves to data (n ≥3) for measurements made 1 min following injection of peptide.

13 without substantially affecting hypertensive potency. D-substitution of the proline residue at position 5 gave the analog with the lowest relative potency of this group perhaps indicative of the importance of this residue in the stabilization of the tertiary structure of NPY through hydrophobic interactions between residues in the polyproline helix and the antiparallel α-helix structure that has been proposed for NPY by Allen *et al.*[7] based on the crystal structure for avian pancreatic polypeptide as solved by Glover *et al.*[8] Several N-terminal D-substitutions, D Tyr^1, D Pro^2 and D Ser^3 resulted in compounds with extended duration of action on MAP. This may be due to stabilization of a conformation which elicits the changes in SVR without the concomitant change in CO. D-substitutions in the C-terminal region of NPY with the exception of Tyr^{36} give analogs with no measurable hypertensive potency at the dose tested. The potency of [D-Tyr^{36}] NPY is, however, very low. The C-terminal carboxamide and the terminal tyrosine residue are required for activity in this assay. The observation that the deletion of the C-terminal amide results in an inactive analog was shown previously for NPY in other assay systems[9,10] and appears to be a common feature of C-terminally amidated bioactive peptides.

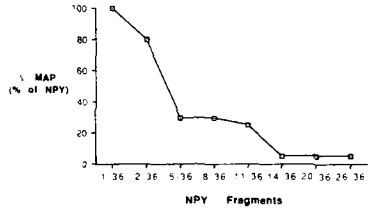

FIGURE 3. Plot of ΔMAP after injection of 0.4 mg/kg NPY and fragments with N-terminal deletions expressed as a percentage of the ΔMAP observed after injection of 0.4 mg/kg NPY ($n \geq 3$).

NPY Fragments

To define the minimum structure analog of NPY for cardiovascular activity we synthesized fragments of NPY with successive deletions of the N-terminus. C-terminally deleted fragments were not synthesized as the requirement of a C-terminally amidated Tyr^{36} residue had already been established. FIGURE 3 shows the results for NPY fragments ranging from 2–36 to 26–36. It is evident that even deletion of the N-terminal tyrosine residue diminishes biopotency as the potency of NPY_{2-36} from comparisons of ED_{50}'s is 0.4 relative to NPY. At 0.4 mg/kg, a dose which elicits a maximal response with NPY, the change in MAP seen at 1 min with NPY_{2-36} is only 80% that seen with NPY suggesting that efficacy is also diminished. For all other fragments efficacy is substantially reduced precluding estimates of relative potency. In order to ascertain the point at which no cardiovascular activity was still measurable we studied NPY $_{16-}$, $_{17-}$, $_{18-}$, $_{19-}$ and $_{20-36}$ at 1.4 mg/kg, a supermaximal dose for the hypertensive activity of NPY. The results of these studies, published elsewhere,[11] suggest that NPY_{18-36} and to a lesser extent NPY_{17-36} elicit a hypotension which is slower in onset and longer lived than the hypertension seen with NPY and many of its 36-residue analogs.

NPY_{18-36}

To redefine this bioactivity in more detail prior to embarking on structure-activity studies for NPY_{18-36} we made measurements of other cardiovascular parameters. These findings have been published and will only be summarized here. The hypotensive action

of NPY_{18-36} is dose related (FIG. 4) and results from the ability of NPY_{18-36} to decrease CO in a manner similar to that seen with NPY without the immediate rise in SVR seen with NPY. Only later, when MAP was reduced was an elevation in SVR observed with NPY_{18-36}. This could be associated with increased plasma levels of epinephrine, norepinephrine, angiotensin II, arginine vasopressin and NPY itself measured 10 minutes following a 1.2 mg/kg dose of NPY_{18-36}.[6] This is likely to represent a reflex compensation for the decreased CO following NPY_{18-36} and may be the source of the late elevation in SVR. These findings suggest that the pharmacophores responsible for the effect on CO and SVR seen with NPY may be defined by the entire structure of NPY and the 18–36 fragment respectively. These differentiated pharmacophores appear to parallel the Y^1 and Y^2 receptor definitions of Schwartz *et al.*[12] which they have suggested are defined by NPY and NPY_{13-36}. Similarly Wahlestedt *et al.*[13] suggested the pre- and postsynaptic differentiation of NPY receptors defined also by NPY and NPY_{13-36}.

NPY_{18-36} Fragments

To define the minimum structure analog for the hypotensive action of NPY_{18-36} we synthesized and tested fragments with N-and C-terminal deletions. The results are summarized in FIGURE 5 and show that at the 0.4-mg/kg dose tested, measurable hypotensive activity is only seen with NPY_{18-36} itself. Deletion of the C-terminal carboxamide results in an inactive compound suggesting a requirement for this moeity, a finding in keeping with that for NPY. The 18–36 fragment appears to the minimum linear structure analog for hypotensive activity.

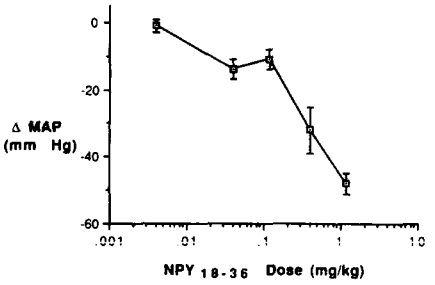

FIGURE 4. Dose-response curve for NPY_{18-36} effect on MAP. Measurements made 5 min following injection of peptide. *Points* represent means ± SEM, n = 11.

NPY_{18-36} Analogs

NPY_{18-36} analogs with D-substitutions throughout the molecule and several with N-terminal modifications were synthesized to probe the structural requirements for hypotensive action at single residues and in the N-terminus. FIGURE 6 shows the results as the change in MAP at 0.4 mg/kg (means ± SEM, $n \geq 3$). The analogs fall into two groups: those with no measurable hypotensive activity at this dose and those with activities comparable to that of NPY_{18-36}. The first group comprise all analogs with modifications at the N-terminus; N-acetylation, N-methylation and desamination. D-substitutions at positions 18,19,20,24 and 25 also do not elict hypotension. This indicates that there is a requirement for a native type conformation at the N-terminus, a finding that contrasts that seen with NPY analogs where modifications to the N-terminus were well

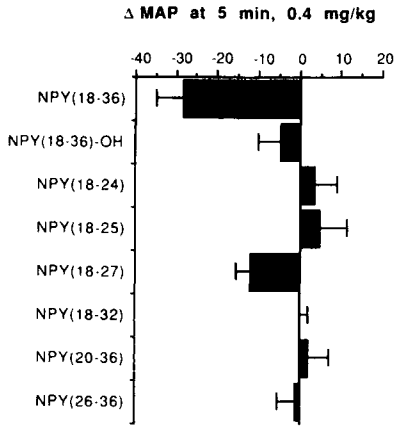

FIGURE 5. ΔMAP for NPY_{18-36} and NPY_{18-36} fragments. Measurements made 5 min following injection of a 0.4 mg/kg dose of peptide. *Bars* represent means ± SEM, n ≥3.

tolerated. NPY_{18-36} analogs which fell into the second group comprised all the D-substituted analogs not listed with the first group. These included NPY_{18-36} analogs with D-substitutions in the C-terminal half of the molecule. This finding also contrasts that seen with NPY analogs where C-terminal D-substitutions (positions 33–36) resulted in inactive compounds. There appears then, to be substantial differences in the structural requirements for the pharmacophore responsible for the hypotension elicited by NPY_{18-36} and certain analogs therof and that for the hypertension elicited by NPY_{1-36} and its analogs.

These findings support the concept of a multiplicity of NPY receptor subclasses which may correlate with the distinct structure-activity relationships for NPY and for NPY_{18-36}.

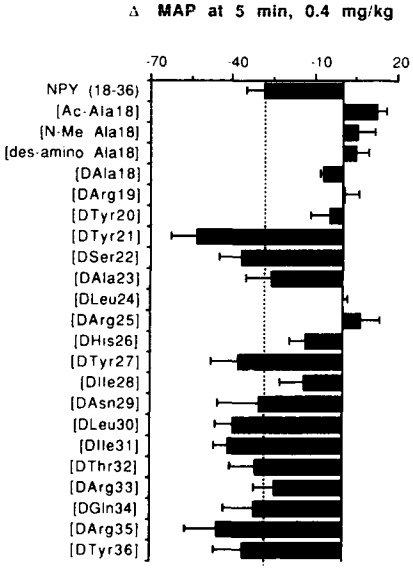

FIGURE 6. ΔMAP for NPY_{18-36}, N-terminally modified and D-substituted NPY_{18-36} analogs. Measurements made 5 min following injection of a 0.4 mg/kg dose of peptide. *Bars* represent means ± SEM, n ≥3.

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Signal Epitopes in the Three-Dimensional Structure of Neuropeptide Y

Interaction with Y_1, Y_2, and Pancreatic Polypeptide Receptors

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INTRODUCTION

It is generally appreciated that regulatory peptides are recognized as three-dimensional structures by the receptors on their target cells. However, our real knowledge of the relationship between the structure and the function of biologically active peptides to a large degree is limited to some information on the importance of certain parts of their primary structure. Although crystal structures are available for a few peptides, for example glucagon, the relevance of these structures for the biologically active conformation of the peptide is unclear because most peptides do not hold a well-defined structure in aqueous solution. Nevertheless, some of these peptides have been shown by nuclear magnetic resonance (NMR) to fold into ordered conformations when the water activity is decreased by organic solvents or in the presence of lipid micelles. Whether these 'artificial' solution structures are of relevance for the receptor-binding conformation of the peptides is an interesting possibility but is still only speculation.

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) are members of the so-called PP-fold family of peptides, which differ from most other small

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and medium size peptides in having a surprisingly rigid conformation in aqueous solution. Furthermore, one member of the family, avian PP, has been characterized in great detail by X-ray crystallography. For the last couple of years, we have studied whether the X-ray structure of PP could be used as a model for the solution structure of NPY and especially whether this folded structure was important for the receptor binding of the peptide. Finally, we are using this structure as a basis for the characterization of the receptor binding epitopes of NPY and PP and for the design of useful analogs in these systems.

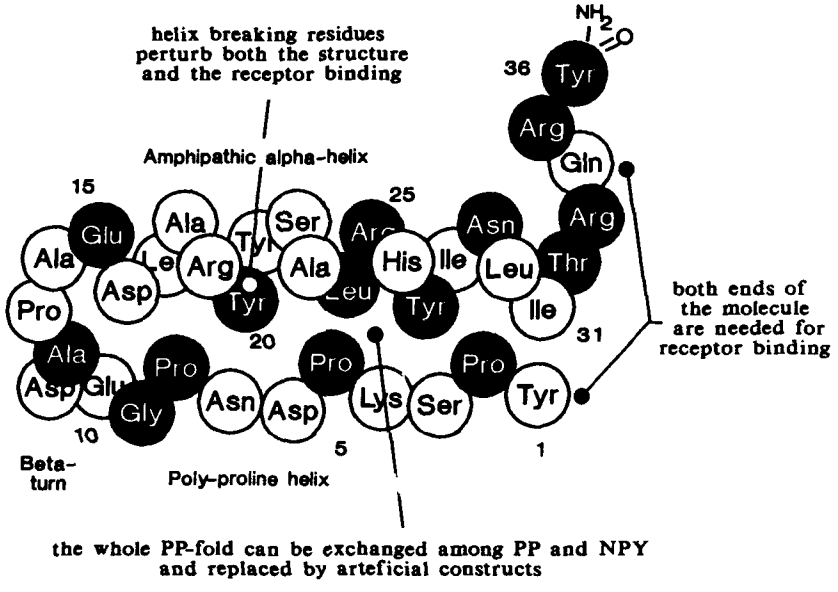

FIGURE 1. Diagram of the structure of NPY indicating the structural elements which have been probed by construction of synthetic analogs in structure-function studies as discussed in the text. The structure has been modeled over the X-ray structure of avian PP.[1] Residues conserved among NPY, PYY, and PP are indicated in *white on black*. Note that these are mainly found in the interdigitating hydrophobic residues in the core of the folded molecule and in the C-terminal pentapeptide, except for the 'discriminating' residue number 34.[25]

The Three-Dimensional Structure of NPY

The Folded X-Ray Structure of PP

In the crystal structure of the 36 amino acid, amidated avian PP, the characteristic PP-fold motif consists of a polyproline-like helix, residues 1–8, and an amphiphilic alpha-helix, residues 15–30, which lie antiparallel with an angle of 152° between the helix axes.[1,2] The two helices are joined by a type I beta turn, residues 9–12, and are held in the folded configuration through hydrophobic interactions between side-chains of the alpha-helix which are interdigitating with the proline residues in the N-terminal section (FIG. 1). Thus, PP-fold peptides appear like small models of large globular proteins in having a well-defined folded configuration with a hydrophobic core and a hydrophilic surface. In the crystal structure, the C-terminal hexapeptide projects away from the

PP-fold at an angle of approximately 90 degrees (see FIG. 1). There is no evident interaction between the main fold of the molecule and the C-terminal segment, which is believed to be important in the receptor interaction (see below). This part of the molecule is also the one which is most flexible in the crystal structure.[3] Thus, very little is known about the conformation of the part of the molecule which interacts with the receptor, despite the fact that the crystal structure is so well characterized.

The overall homology among members of the PP-fold family within a given species varies between 45 and 70%. More importantly, however, as shown in FIG. 1, the identical residues are mainly the core residues which are important for the stabilization of the PP-fold as such, and the residues in the C-terminal end.[4,5] Among the over 25 different PP-fold peptides characterized today from different species, there is also a strong conservation of the core residues, whereas the surface residues vary rather freely among hydrophilic residues.[5] Thus, NPY, PYY, and PP appear to be peptides with a common folded structure but in which the exposed surface differs in detail, although remaining hydrophilic in nature.

PP-Fold Peptides Have Ordered Structure in Solution

Circular dichroism (CD) studies of different PP-fold peptides indicate that they all have a considerable amount of secondary structure in aqueous solution.[6–11] This is even true for the monomeric form of NPY.[9] Initially, the lack of a suitable reference spectrum for the polyproline-like helix prevented a detailed computational analysis of the spectra.[9] However, the calculated difference spectrum between the experimental spectrum of NPY and that of an 8–36 fragment of NPY, lacking the N-terminal polyproline part of the structure, was similar both in shape and intensity to the CD spectrum of collagen.[10] This observation indicates that in aqueous solution the N-terminal part of NPY adopts a polyproline-like structure equivalent to the helical structure of collagen and similar to that found in the crystal structure of avian PP. By using this difference spectrum as a reference spectrum for the polyproline-like helical structure, computational analysis of the CD spectrum for NPY gave results which closely resembled those of the PP crystal structure.[10] Thus the CD analysis supports the idea that in aqueous solution NPY contains secondary structural elements similar to those found in the tertiary structure of the PP-fold.

Two-dimensional NMR studies have been performed on NPY in aqueous solution.[12] In these studies, performed at pH 3.1, a long alpha-helical segment from residue 11 through to the C-terminus was clearly assigned. The N-terminal segment, which is in the polyproline-like helix in the crystal structure of avian PP, did not assume any regular structure which was indicated by the observation that the prolines were in both cis and trans conformations.[12] Unfortunately these studies could not be performed at physiological pH due to solubility problems. However, by CD analysis an increase in the ordered structure was observed when the pH was increased to 7.4, but this was not interpreted to be a result of the formation of a polyproline-like helix, but rather as a further extension of the alpha-helical segment.[12] Thus, although the 2-D NMR data indicate that NPY does have a relatively large amount of ordered structure in aqueous solution, they do not support the notion that NPY has a folded structure similar to the X-ray structure of avian PP.

Proteolytic stability of the PP-fold. Evidence in favor of the presence of a rigid, folded structure of these peptides in solution comes from studies on their stability towards proteolytic degradation. In earlier studies it was noticed that the hormone PP was surprisingly stabile in blood samples as compared to other biologically active peptides.[13] As shown in FIG. 2, it takes more than 20 hours to cleave intact PP with an enzyme, for

example, protease Asp-N, which cleaves a sequence found in the presumed beta-turn of the molecule. However, under identical conditions this sequence is cleaved within half an hour in a long fragment of PP, which lacks part of the sequence which stabilizes the PP-fold in the crystal structure.[13] These results, together with those obtained with endoprotease Glu-C and with NPY and full-length, un-folded NPY analogs, indicate that in solution a large fraction of the molecules of PP-fold peptides must be found in a configuration which is not accessible for the enzymes.

In conclusion, all studies to date show that NPY and the other PP-fold peptides have a surprisingly high degree of secondary structure in aqueous solution as compared to most other peptides of similar size. However, it is not quite clear whether under physiological conditions the solution structure of these peptides is equivalent to the well-characterized crystal structure of avian PP; although there is some evidence that this is in fact the case.

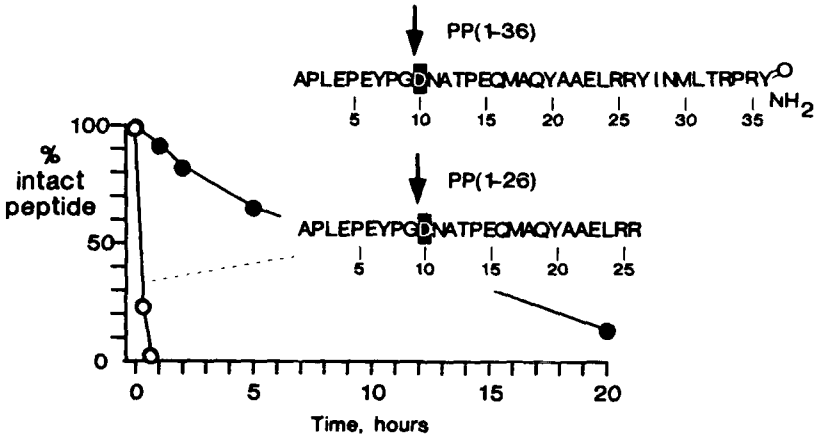

FIGURE 2. Proteolytic stability of intact, folded PP compared to the unfolded, long N-terminal fragment PP^{1-26}. The digestion of PP^{1-36} and PP^{1-26} with endoproteinase Asp-N was performed in 0.01 M Tris/HCl buffer, pH 7.5, at room temperature. The products were separated by high performance liquid chromatography and identified by amino acid analysis. The identical cleavage site in both peptides is indicated by an *arrow*; in the crystal structure this site is situated in a beta-turn (see FIG. 1). Similar experiments have been performed with NPY and PP-fold perturbed NPY analogs (O'Hare and Schwartz, to be published). (The figure is redrawn from REF. 13.)

Probing the Importance of the 3D-Structure for the Receptor Binding of NPY

There Are Two Types of NPY Receptors

Receptors for PP-fold peptides have been characterized in neuronal tissues,[14-17] on neuronal cell lines,[16,18] on epithelial cells in the intestine and kidney,[19,20] and on vascular smooth muscles.[21-23] Apparently, NPY and PYY share common binding sites, which have been subdivided into two classes, Y_1 and Y_2. This subdivision was originally based on bioassays[24] and ligand binding studies[5,16] using long C-terminal fragments of NPY or PYY which selectively reacted with the Y_2 type of receptors. Recently we have designed an analog, [Leu^{31},Pro^{34}]NPY, which selectively reacts with the Y_1 type of receptor.[25,26]

Thus today, the two types of NPY/PYY receptors can be defined through opposite binding profiles for two or more analogs, in analogy with the opioid receptors. Cross-linking experiments have demonstrated that Y_1 and Y_2 receptors are not only pharmacologically different entities but also structurally distinct glycoproteins.[27] Although there are certain tissue and species differences, a simplified picture of the physiological importance of the two different types of NPY/PYY receptors is that the Y_1 receptors are mainly postjunctional receptors involved in vasoconstriction and that Y_2 receptors are mainly prejunctional receptors in the central and peripheral nervous system. Y_2 receptors also function as postjunctional receptors in epithelial cells in the intestine and the kidney, as well as in some vascular systems. Receptors which selectively react with PP and not with NPY/PYY have been identified on a neuronal cell line,[18] on intestinal epithelial cells,[28] and in certain specific brain areas such as the area postrema.[29]

As presented above, both spectroscopical data and classical biochemical data indicate that NPY and the other PP-fold peptides probably hold structures in solution which are similar to each other and conceivably analogous to the crystal structure of avian PP. The question which we have addressed is whether this structure is important for the biological function of the peptides. *According to our current model, the PP-fold mainly functions as a structural element which brings the C- and N-termini of the molecule in close contact and presents the combined moiety to the receptor.*[5,10,30] This model has been tested through a series of different approaches with synthetic peptide analogs in ligand binding assays. In these studies we have mainly used human Y_1 and Y_2 receptor-assays based on the convenient, selective expression of these receptors on human neuroblastoma cell lines or porcine hippocampal membranes with Y_2 receptors.[5,16]

Fragments of NPY Indicate that Both Y_1 and Y_2 Receptors Need Both Ends of NPY for Binding

Although the C-terminal, amidated ends of both NPY and PP are essential for binding and function of the peptides,[24,31] C-terminal fragments of NPY have to be at least 16 residues long, just to give approximately 10 percent of the activity of intact NPY on the Y_2 type of receptor.[10] Furthermore, the binding affinity is not increased above this point until the residues in the far N-terminal end of the molecule are added to the long C-terminal fragments. On the Y_1 receptors, no significant binding is obtained at all before the far N-terminal part of the molecule is included in the analogs.[10] In other words, on both types of NPY receptors, both ends of the molecule are needed for full binding activity. In the crystal structure of avian PP, these two ends are brought in close proximity by the PP-fold structure.

Perturbation of the PP-Fold Perturbs Receptor Binding

In order to 'disprove' our hypothesis, we tried to prevent the NPY molecule from folding into the hypothetical conformation using only a single substitution. Due to the special rigid bonds in the amino acid proline, this residue is known to be helix-breaking and never to occur in alpha-helices, except occasionally among the first three residues.[32] Thus, we decided to try to disrupt the PP-fold in NPY by introducing a single proline residue at position number 20, in the middle of the supposed alpha-helical region. This substitution had the desired effect on the solution structure, as almost no helical signal was observed in the circular dichroic spectrum of the analog as opposed to the clear helical signal observed with authentic NPY.[10] As shown in FIG. 4A, this single substitution shifted the binding curve in Y_2 receptors to a position corresponding to that of a long

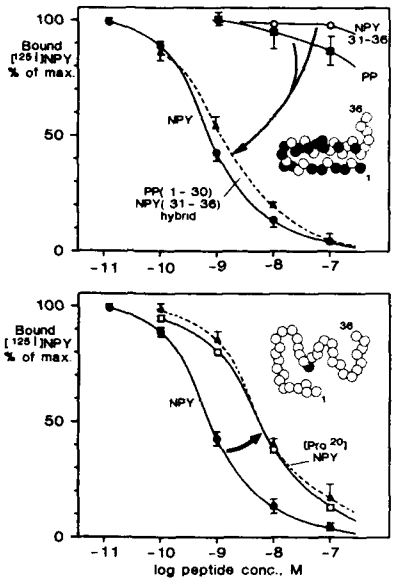

FIGURE 3. Probing the importance of the PP-fold for NPY receptor binding. *Lower panel:* the effect of unfolding the PP-fold in NPY on Y_2 receptors. The PP-fold of NPY was disturbed by introducing a single helix-breaking proline residue. The displacement of monoiodinated NPY from porcine hippocampal membranes by NPY, [Pro^{20}]NPY, and NPY(19–36)peptide is shown. The *inset* to the right indicates that [Pro^{20}]NPY is found in almost random coil in contrast to normal NPY according to circular dichroism studies (Fuhlendorf *et al.*, 1990b).[10] The analog does not bind at all to Y_1 receptors. *Upper panel:* the lack of effect of swapping the PP-fold motif from PP to NPY on Y_2 receptors. By placing the C-terminal hexapeptide from NPY on the PP-fold of PP an analog is created which reacts equally well as NPY on porcine hippocampal Y_2 receptors. The *arrows* indicate the change in activity gained by combining the two building blocks, NPY(31–36)peptide and PP, which by themselves are devoid of activity in this system. In the *inset* to the right the many differences in the structure of the hybrid analog compared to NPY are shown in *black*.

C-terminal fragment. Thus, the intact N-terminal part of the analog was not recognized by the receptor. On the Y_1 receptors the analog did not bind at all.[10] These observations support the hypothesis that the secondary structure is important for the receptor binding of NPY.

The PP-Fold Motif Can Be Exchanged Freely among Different PP-Fold Peptides

Kaiser and Kèzdy have made an impressive case for the importance of secondary structural elements in the function of medium size peptides.[33,34] They have designed an experimental approach for such studies in which they replace a segment of a peptide with a nonhomologous segment which preserves the secondary structure.[35–37] In this way, evidence has been provided for the functional importance of segments of amphiphilic secondary structure in the binding and action of, *e.g.*, endorphine and calcitonin. This was achived by minimizing the sequence homology of the analogs, even including non-peptide elements, and, for example, exchanging a suspected amphiphilic alpha helix with an amphiphilic helix composed of D-amino acids.[36–38] When this approach was applied to NPY, the peptide analogs did have the desired physicochemical properties; however, in biological terms the analogs reacted in the best case with less than one percent of the potency of NPY.[8] It should be emphasized that PP-fold peptides appear to differ from endorphin and calcitonin in holding a globular, tertiary structure in aqueous solution; a structure, in which the amphiphilic helix is stabilized through intramolecular, hydrophobic interactions (FIG. 1). Therefore, we decided to apply the methodological approach of Kaiser and Kèzdy in a modified form. The structural motif to be probed, the PP-fold, was swapped between NPY and PP. In other words, we used a motif of secondary structure, which is vastly different in sequence, but which has been structurally refined through the evolutionary process.[5] The hybrid analog and authentic NPY bound equally well to Y_2 receptors, despite the fact that the two components of the hybrid, PP and the C-terminal

segment of NPY, by themselves did not bind significantly (FIG. 3B).[10] Thus, the PP-fold motif can apparently be relatively freely exchanged between different members of the family, which strongly indicates that this part of the molecule has a purely structural role.

The PP-Fold Can Be Substituted with a Nonpeptide, Bridging Construct

If the hypothesis is correct, that the PP-fold mainly functions to bring the C- and N-terminal segments of the molecule closely together, then it should be possible to exchange the fold, not only with a nonhomologous peptide-fold but also with a nonpeptide type of chemical construct. Such NPY analogs, in which the PP-fold was replaced with chemical bridges at different positions, with and without stabilizing disulfide bridges, have been created through computer-aided molecular design.[30,39] Krstenansky and co-workers produced centrally truncated NPY analogs where, *e.g.*, residues 8–17 were exchanged with an 8-aminooctanoic acid residue and a stabilizing disulfide bridge was placed between residues 7 and 20. These analogs reacted equally well as NPY with NPY receptors in mouse brain membranes.[30] Beck and collaborators went even further and replaced residues 5–24 with a single epsilon-aminocaproic acid spacer. This analog had an affinity of one third of NPY on brain NPY receptors and had a potency of one tenth of NPY in the vas deferens.[39] This surprisingly high potency of the analog on the Y_2 type of receptors was not found in the presynaptic, blood pressure assay.[39] We have recently confirmed this with a similarly truncated, but disulfide-stabilized NPY analog (FIG. 4), which reacts equally well as NPY on human Y_2 receptors, but very poorly with Y_1 receptors (Vervelde, Krstenansky, and Schwartz, to be published). This short analog also stimulates chloride absorption in intestinal epithelial cells with a potency not much less than NPY (Cox and Krstenansky, to be published). Thus, in respect of reactivity on the Y_2 receptor the PP-fold can be exchanged with different nonpeptide constructs, provided that the close spatial orientation of the N- and C-terminal ends of the molecule are retained. This is not the case for the Y_1 receptor.

Defining the Differential Specificity of PP-Fold Receptors Based on the 3-D Structure of NPY/PP

Residues Which Determine that Receptors Recognize NPY and PP Differently Are Found in the Discontinuous Epitope Presented by the PP-Fold

The studies described above and especially the fact that full binding activity can be obtained on the Y_2 receptor with an analog in which residues 5–24 have been replaced by

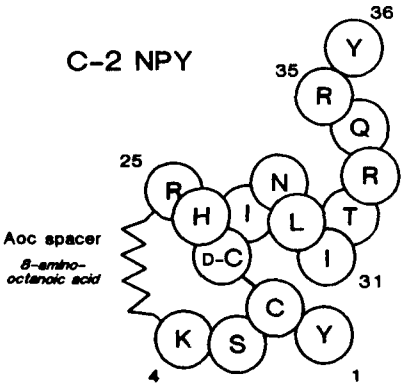

FIGURE 4. Simplified diagram of a centrally truncated and disulfide stabilized NPY analog, C-2 NPY, which reacts equally well as NPY/PYY on Y_2 receptors (Vervelde, Krstenansky, and Schwartz, to be published).

a simple, nonpeptide bridge, demonstrate that the epitope which binds to the receptor is composed of residues found in the combined C- and N-termini of the molecule.

As shown in FIG. 5, nine residues are common among all three known PP-fold peptides in man within this discontinuous epitope. Systematic substitutions with D-amino acids along the NPY sequence have shown that residues 32–34 in particular are crucial for binding to receptors on brain membranes, conceivably Y_2 receptors (Boublik, this volume). Binding studies with a series of hexapeptides corresponding to the C-terminal end of NPY in some avian PP/NPY receptors in chicken cerebellum identified the guanidino moiety of the arginine residue in position 35 as a major receptor-binding element.[41] Thus, the side chains of some of the common residues of NPY/PYY and PP, indicated by grey symbols in FIG. 5, are involved in receptor-ligand interactions. However, one or more of the side chains of the residues which differ between NPY/PYY and PP, shown in white-on-black in FIG. 5, must determine the very different affinities with which these homologous peptides are recognized by the different receptors for PP-fold peptides. In other words, among these residues we can find the key to the different receptors. Some of the substitutions are rather conservative whereas others are more chemically significant. In

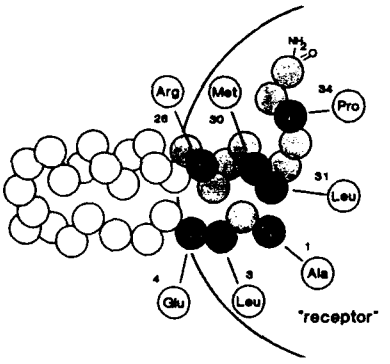

FIGURE 5. Differences between NPY/PYY and PP in the hypothetical receptor binding epitope of the ligand molecules. A very simplified 'model' of a receptor is shown to the *right*. The *grey symbols* represent the residues which are identical between NPY, PYY, and PP, whereas the residues found in NPY/PYY are indicated in *white on black*. The corresponding residues found in PP are shown around the model of NPY/PYY. The structural basis for the differential recognition of NPY/PYY and PP by NPY/PYY and PP receptors should be found in these different residues. Residue number 34 is important for the differential recognition of NPY/PYY and PP by the Y_2 and the PP receptor but not the Y_1 receptor.[25,41] Residue number 4 is important for the differential recognition by the Y_1 and the PP receptor (to be published).

order to delineate the structural background for the differential specificity of Y_1, Y_2, and PP receptors, we have systematically exchanged the residues shown in black in FIG. 5; *i.e.*, gradually made the NPY molecule PP-like and in a parallel series of analogs gradually made the PP molecule more NPY-like.

Residue 34 Is the Key to the Differential Recognition of NPY/PYY and PP by Y_2 and PP receptors but Not by Y_1 Receptors

Since residue number 34 is only one which differs among the last 5 residues in NPY/PYY and PP, and since the difference is major, a glutamine residues in NPY/PYY and a proline in PP, this site was chosen as the initial target. In the first analogs of this series, residue number 31, Ile in NPY and Leu in PP, was substituted along with residue number 34. As shown in FIG. 6, NPY Y_2 receptors, which normally do not bind PP, recognize this hormone equally well as NPY if only residues 31 and 34 are exchanged with those of NPY. Conversely, [Leu^{31},Pro^{34}]NPY is recognized equally poorly as PP by

the Y_2 receptors. In fact, the binding of NPY to Y_2 receptors is upset just by the single substitution of Gln to Pro in position 34 (42). Thus, residue number 34 is the key to the differential binding of NPY/PYY and PP to Y_2 receptors. If this residue is a glutamine the peptide binds and activates the receptor, if it is a proline it does not. This is not at all the case for Y_1 receptors. Exchange of residue 34 (and 31) between NPY and PP has no effect on the binding of the peptides to Y_1 receptors (FIG. 6).[25] Thus, the far C-terminal segment of the molecules is not involved when a Y_1 receptor discriminates between NPY/PYY and PP. Nevertheless, the Y_1 receptor does not welcome any type of side chain in position 34 of the PP-fold molecule. For example, the imidazole ring of histidine and the large hydrophobic aliphatic side chain of isoleucine are not allowed (Kjems, Kristensen, Krstenansky, and Schwartz, to be published).

On the PP receptors, the substitutions in the C-terminal hexapaptide had major impact on the binding affinity. The introduction of proline in position 34 and leucine in position 31 increased the binding affinity of NPY by several orders of magnitude (FIG. 6). Similarly, but in the opposite direction, substitutions in the PP molecule in positions 31 and 34 with the NPY residues decreased the binding affinity by more than two orders of magnitude (FIG. 6).

Residue 4 Is Important for the Differential Recognition of NPY/PYY and PP by Y_1 and PP Receptors

Among the other residues which differ between NPY/PYY and PP, we have found that substitution of lysine in position 4 of NPY with glutamic acid (as in PP) in an analog also carrying a proline residue in position 34 increases the binding of the molecule to PP receptors. In addition, this analog, [Glu^4,Pro^{34}]NPY, binds with less affinity to Y_1 receptors as compared to NPY and [Pro^{34}]NPY (Fuhlendorff, Kristensen, Kjems, and Schwartz, to be published). These preliminary data, indicate that residue number 4 in the ligands is involved in the determination of the differential specificity of Y_2 and PP receptors.

General Importance of the PP-Fold Motif

The PP-fold is important in bringing the C- and N-terminal parts of the ligand together and presenting the discontinuous epitope to the receptors. However, certain physicochemical characteristics of the PP-fold indicate that this moiety may be more "actively" involved in the ligand-receptor interaction process.

The PP-Fold Motif Is Amphiphilic across the Axis

The alpha-helical segment of all PP-fold molecules is strongly amphiphilic. Although a major part of the hydrophobic face of the helix is buried in the core of the molecule (see above), the PP-fold as such presents a longitudinal, hydrophobic patch along the axis of the fold (FIG. 7). This hydrophobic patch is involved in dimerization of the molecules as characterized in avian PP crystals. As discussed above, in several medium-size peptides an amphiphilic segment of the structure is involved in the binding of the ligand to its receptor in a purely structural way.[33,34] We are currently investigating whether the hydrophobic patch along the axis of the PP-fold of NPY is important for binding of the peptide to Y_1 and Y_2 receptors.

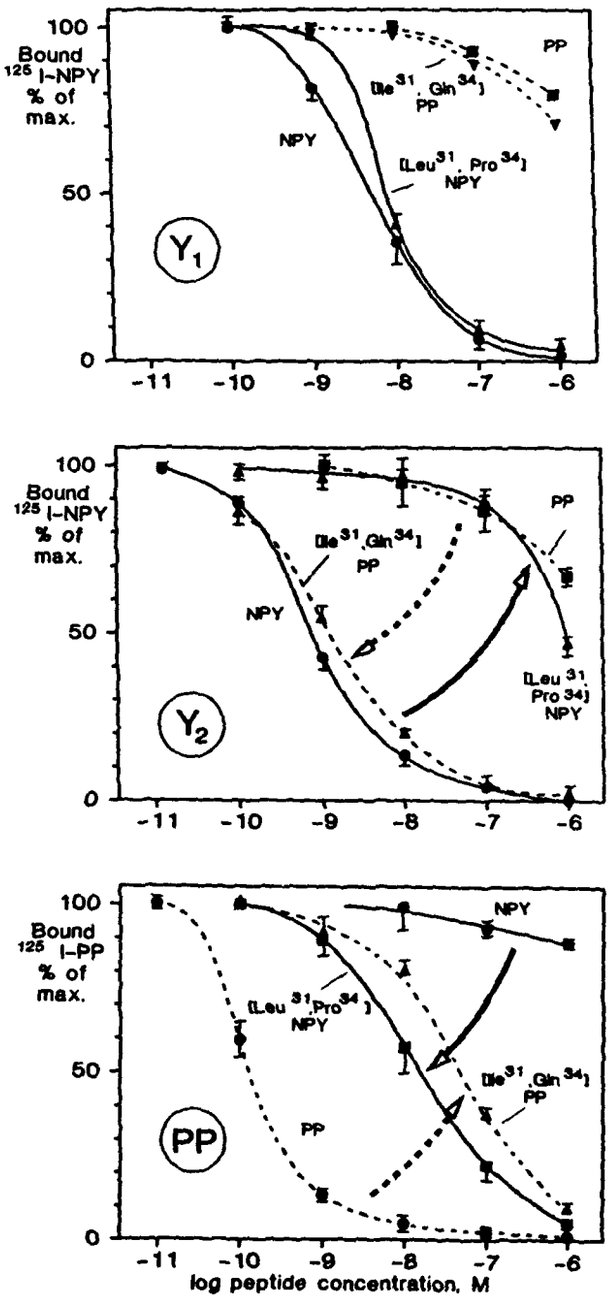

FIGURE 6. Delineation of the structural basis for the differential specificity of receptors for PP-fold peptides. Binding of NPY, PP and corresponding hybrid analogs to NPY Y_1, Y_2 and PP receptors. Note that it is possible to totally change the binding of PP and NPY to Y_2 receptors just by substituting residues 31 and 34, of which residue number 34 is the important key, and that these substitutions have no effect on binding to Y_1 receptors (see text). In this case, the Y_1 receptors and PP receptors are on PC 12 cells and Y_2 receptors on porcine hippocampal membranes (redrawn from REF. 41).

PP-Fold Peptides Have a Strong Electrostatic Dipolar Moment along the Axis of the PP-Fold

In PP-fold peptides there is a clear spatial segregation of positively charged groups in the combined C- and N-terminal epitope and negatively charged amino acid residues in the turn region of the PP-fold (see FIG. 1). This electrostatic dipolar moment has been well conserved during evolution although the type and exact location of the negatively charged residues in particular has varied (Bjørnholm and Schwartz, to be published). The dipolar moment could be instrumental both in the stabilization of the peptide structure and more directly in receptor binding of the receptor. Capping of alpha-helices with acidic residues at the N-terminus and basic residues at the C-terminal end is important for the helical stability in general[43] and could therefore be important in stabilizing the PP-fold. Recently is was noted that the free acid form of NPY, which is devoid of any biologicaly activity, is unstable in solution with respect to secondary structure.[11] In this molecule, the deamidation in the positively charged pole of the molecule has an effect on receptor binding which is energetically much more dramatic than can be explained by the disruption of an interaction between the amide group and the receptor alone. Destabilization of the alpha-helix and thereby destabilization of the whole PP-fold structure could better explain the striking impotency of the free acid form of NPY and of other PP-fold peptides. We are

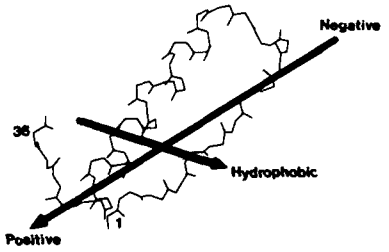

FIGURE 7. Model of NPY showing only the backbone structure. The strong electrostatic dipolar moment and the direction of the amphiphilic moment are indicated by *arrows*.

currently investigating the effect on structural stability and biological effect of changes in the size and direction of the dipolar moment in NPY through substitutions in the acidic part of the PP-fold, distant from the proposed receptor binding epitope of the Y_2 receptor, at least.

CONCLUSION

NPY has in aqueous solution a stable structure characterized by a high degree of secondary structure which could be equivalent to the PP-fold characterized in detail in crystals of the homologous avian PP. Studies with synthetic peptide analogs have shown that the tertiary structure of NPY is very important for the receptor binding of the peptide to both Y_1 and Y_2 receptors. The PP-fold appears to function mainly to bring the C- and N-terminal segments of the molecule in close proximity and present the combined moiety to the receptors. Thus, the PP-fold motif can freely be exchanged among PP-fold peptides and can even be exchanged by a nonpeptide, bridging construct. In the receptor-binding epitope of PP-fold peptides, residue number 34 defines the differential specificity of Y_2 receptors. By changing just this single residue in PP from Pro to Gln as in NPY, the PP-analog is recognized equally well as NPY; conversely, by changing Gln^{34} in NPY to

Pro, the NPY analog looses all binding activity in Y_2 receptors. In NPY as in all other PP-fold peptides, there is a strong electrostatic dipolar moment along the axis of the motif as well as an amphiphilic moment across the motif. It is suggested that these physicochemical characteristics are important both for the structure and function of the peptides.

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Comparison of the Neuropeptide Y Receptor in the Rat Brain and Intestine[a,b]

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INTRODUCTION

Neuropeptide Y (NPY), together with pancreatic polypeptide (PP) and peptide YY (PYY), constitute a family of structurally related peptides all of which contain 36 amino acids and have a similar tertiary structure.[1-4] NPY has neurotransmitter and neuromodulator functions in the central, peripheral and enteric nervous systems.[5-7] Central administration of NPY increases food intake,[5] produces hypotension, bradypnea and EEG synchronization,[6] and shifts circadian rhythms.[7] NPY applied to intestinal mucosa mounted in Ussing chambers inhibits ion transport with an EC_{50} of 10–30 nM;[8,11] it inhibits transmural electrical potential and short circuit current, increases mucosal-to-serosal Na^+ and Cl^- fluxes, and reduces serosal-to-mucosal Cl^- fluxes.[8-11] This inhibitory action is only demonstrable when NPY is added to the serosal (but not luminal) side of the intestinal epithelium. These observations correlate with the localization of NPY to intrinsic nerves within the enteric nervous system that end in close proximity to the laterobasal region of the intestinal epithelial cell.

We have used a recently described method that allowed isolation of intestinal serosal latero-basal membranes (LBM) free from contamination with luminal brush border membranes (BBM), and intracellular endoplasmic reticulum (ER) and Golgi vesicles[12] to demonstrate that NPY binds preferentially to the serosal LBM of the enterocyte. In addition we have cross-linked radiolabelled NPY to its intestinal receptor, and compared the resulting complexes observed on SDS-polyacrylamide gel electrophoresis with the pattern observed after cross-linking the brain receptor.

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[b]Abbreviations used: NPY: neuropeptide Y; PYY: peptide YY; PP: pancreatic polypeptide; HPLC: high performance liquid chromatography; ER: endoplasmic reticulum; LBM: lateral membranes; BBM: brush border membranes; HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BSA: bovine serum albumin; DTT: dithiothreitol; SDS: sodium dodecyl sulfate; PMSF: phenylmethylsulfonyl fluoride; DSS: disuccinimido suberate; EDTA: ethylenediaminetetraacetic acid; PAGE: polyacrylamide gel electrophoresis; lacto-NPY_1: lactoperoxidase labelled NPY contained in the first radioactive peak isolated on HPLC; IODO-GEN-NPY_2: IODO-GEN labelled NPY contained in the second radioactive peak isolated on HPLC; B-H-NPY: Bolton-Hunter labelled NPY.

METHODS

Chemicals and Reagents

Synthetic NPY, PYY and PP were purchased from Peninsula Laboratories (Belmont, CA). Bolton-Hunter labelled [^{125}I-Lys^4] NPY (BH-NPY) was purchased from New England Nuclear (specific activity 2200 Ci/mmol). For some experiments one or more of the five tyrosines in NPY was radioiodinated using lactoperoxidase[13] or IODO-GEN,[14] and the resulting radiolabelled species purified on high performance chromatography (HPLC).[14] Lactoperoxidase was purchased from Calbiochem (Los Angeles, CA), Na^{125}I from Amersham Corp. (Arlington Heights, IL), polyacrylamide gel electrophoresis reagents from Bio-Rad (Richmond, CA) and molecular weight standards for gel electrophoresis from Pharmacia (Pistcataway, NJ). Triton-X-100 was from J.T. Baker (Phillipsburg, NJ), and all the protease inhibitors (antipain, chymostatin, pepstatin, leupeptin, phenylmethylsufonyl fluoride (PMSF), ovomucoid trypsin inhibitor, bacitracin, aprotinin) were from Sigma. The crosslinking agent disuccinimido suberate (DSS) was purchased from Pierce (Rockford, IL). X-ray film (XAR-2) was from Eastman Kodak (Rochester, NY). All other chemicals used were of reagent grade.

Intestinal Cell Membrane Fractionation

Membranes were prepared from intestinal epithelial cells dissociated from the jejunum and ileum of fasted male Sprague Dawley rats (~300 gm) using previously described methods.[12] Protease inhibitors were added during cell dissociation (2.5 μg/ml each of antipain, chymostatin, pepstatin, leupeptin, ovomucoid trypsin inhibitor, and 1 mM PMSF) and during the first homogenization (25 μg/ml each of antipain, chymostatin, pepstatin, leupeptin, ovomucoid trypsin inhibitor, and 1 mM PMSF). Differential centrifugation was used to yield a pellet (P_3) that contained mostly ER-Golgi and LBM; precipitation of ER-Golgi with 8 mM $CaCl_2$ combined with differential centrifugation yielded a pellet (P_B) that contained mostly LBM and BBM. The membrane populations contained in P_3 and P_B were subsequently separated by centrifugation (85,000 x g for 14–16 hr) in linear sorbitol gradients (25–60% w/v). Fractions (2 ml) were collected from the top of the gradient and assayed for protein (Coomassie blue dye binding using reagents obtained from Bio-Rad), marker enzymes (aryl esterase for ER-Golgi; K-stimulated phosphatase for LBM; sucrase for BBM), and for NPY binding. In other experiments, all the membranes contained in the whole homogenate were subjected to calcium precipitation to increase the yield of LBM.

Binding of NPY to Membrane Fractions

Aliquots (200 μl) of the gradient fractions derived from the P_B pellet were incubated for 1 hr at room temperature with trace amounts (10–50 pM) of labelled NPY dissolved in 800 μl of 10 mM NaH_2PO_4-K_2HPO_4, 0.2 TIU/ml aprotinin, 0.5% bovine serum albumin (BSA), 0.05% bacitracin, pH 7.0. When NPY was labelled with lactoperoxidase or iodogen, Triton X-100 was added to the incubation buffer at a final concentration of 0.006% to minimize the nonspecific binding of labelled peptide to the polypropylene assay tubes. At the end of the incubation period, the membrane-bound ligand was separated by centrifugation at 27,500 x g for 30 min at 4°C. Total radioactivity at the beginning of the experiment and in the membrane pellet was determined and specific binding corrected for nonspecific binding (number of apparently bound counts observed

in the presence of 1 μM unlabelled NPY.) Scatchard analysis was performed using the EBDA/LIGAND program (originally written by P. J. Munson and D. Rodbard and modified by G. A. McPherson) obtained commercially from Biosoft/Elsevier (Milltown, NJ).

Cross-Linking of NPY to Intestinal Membranes

Membrane fractions were pooled according to their enzyme markers, washed twice, resuspended to a concentration of 1 mg/ml of membrane protein in 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 5 mM histidine-imidazole, pH 7.4. The membrane pools were frozen in liquid nitrogen, and stored at −70°C. Binding studies were performed as in the prior section except that 100 μg of membrane protein was used for each assay. The membrane pellet obtained after allowing radiolabelled NPY to bind was washed (centrifugation at 27,500 x g for 30 min) and finely resuspended with a 25 gauge needle in 1 ml of 60 mM HEPES, pH 7.5. Cross-linking was initiated by the addition of 10 μl of 20 mM DSS dissolved in dimethyl sulfoxide (final concentration ~0.2 mM), and the membranes were then incubated for 15 min at 4°C on a rotary shaker. The crosslinking was stopped by the addition of 2 ml of cold 60 mM HEPES, 60 mM ammonium acetate, pH 7.5. The membranes were centrifuged at 27,500 x g for 30 min and washed with 62.5 mM Tris-HCl, pH 6.8, prior to SDS polyacrylamide gel electrophoresis.

Preparation of Brain Membranes, NPY Binding, and Cross-Linking

Male Sprague-Dawley rats (200–250 g) were anesthetized with intraperitoneal pentobarbital, decapitated and the brains rapidly removed. The cerebellum and white matter were dissected away from the cortex, and the cortex then homogenized with a glass-on-glass Dounce homogenizer (6 passes pestle B, 10 passes pestle A) in 10 ml of ice-cold "Buffer A" (137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 0.1% bacitracin, 0.1% glucose, 0.2 TIU/ml aprotinin and 20 mM HEPES, pH 7.4). The homogenate was brought to a volume of 30 ml with "Buffer A" and centrifuged at 14,000 x g and 4°C for 15 min. The resulting pellet was washed and resuspended in a final volume of 4.5 ml buffer A. Binding and cross-linking experiments were performed using the same methods described above for the intestinal membrane except that binding was performed in "buffer A" supplemented with 0.005% Triton X-100 and 0.2% BSA and cross-linking was performed with 1 mM DSS.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

Cross-linked membrane pellets were resuspended in 150 μl of a solubilization buffer containing 2% SDS, 10% w/v glycerol, 1 mM EDTA, 10 mM DTT, bromphenol blue and pyronin Y as dye indicators, and 0.0625 M Tris-HCl, pH 6.8; DTT was omitted when using DSP. Following incubation in a shaking water bath at 37°C for 30 min, the resulting membrane suspension, or the solubilized material contained in the supernatant after centrifugation at 18,000 x g and 22°C for 5 minutes was studied by 10% polyacrylamide gel electrophoresis and autoradiography as outlined by Nguyen *et al.*[15,16] In some experiments, radioactive bands were cut from the dried gels using the autoradiogram as a template and counted directly.

RESULTS

Binding of NPY to Pooled Membrane Fractions

Specific binding of Bolton-Hunter labelled NPY to LBM (40 μg of membrane protein) was 5.8% ± 1.4% of the total radioactive NPY added. Binding of NPY to LBM exhibited dependence upon time and the amount of membrane protein added. In contrast the nonspecific binding to BBM observed in the presence of 10 μM NPY was actually higher than the total binding in the absence of unlabelled NPY. Half-maximal inhibition of labelled NPY binding to LBM was observed with 20–50 nM unlabelled NPY. Scatchard analysis demonstrated equally good fits ($P > 0.05$) for a single binding site model (K_D 15 nM, B_{max} 30 pmole/mg of membrane protein) and a two-binding-site model (K_D 5.3 nM, B_{max} 4 pmole/mg of membrane protein [Site 1]; K_D 29 nM, B_{max} 36 pmole/mg of membrane protein [Site 2]).

When NPY was radiolabeled with lactoperoxidase or IODO-GEN and purified by HPLC, at least 3 major peaks corresponding to different sites of iodination were observed (NPY has 5 tyrosine residues that can potentially be iodinated). Sheikh *et al.*[14] previously demonstrated that, following IODO-GEN radioiodination, NPY contained in the first radioactive peak (IODO-GEN-NPY_1) on HPLC was monoiodinated at tyrosine residue 1; NPY contained in the second radioactive peak (IODO-GEN-NPY_2) was monoiodinated at tyrosine residue 36; and NPY contained in the third major peak or radioactivity (IODO-GEN-NPY_3) was iodinated at both positions 1 and 36.

We determined whether the preferential binding of radiolabeled NPY to LBM was dependent on site of iodination of the ligand. IODO-GEN-NPY_1 bound well to LBM (11% of the initial amount of NPY added specifically bound to 100 μg of membrane protein) but poorly to BBM (1% specific binding to *100 μg of membrane protein). In* contrast, IODO-GEN-NPY_2 and IODO-GEN-NPY_3 both bound well to LBM (20–25% specific binding to 100 μg of membrane protein), but also BBM (8–10% of the initial amount of NPY bound specifically to 100 μg of membrane protein). The lactoperoxidase-labeled NPY obtained from the first peak of radioactivity observed on HPLC (lacto-NPY_1) was assessed in the same manner. The specific binding of NPY_1 to LBM and BBM (75 μg of membrane protein) was respectively 14.8% ± 1.3% and 1.3% ± 0.1% of the initial amount of radioactive NPY_1 added. Since IODO-GEN-NPY_2 and IODO-GEN-NPY_3 are both radioiodinated at position 36, it is possible that this is the common feature that allows demonstration of ligand binding to BBM.

Assessment of Label Degradation

As mentioned above, NPY radioiodinated either at the fourth lysine residue using the Bolton-Hunter agent (B-H-NPY_4) or at the first tyrosine residue (NPY_1) using either IODO-GEN or lactoperoxidase binds specifically to LBM but not to BBM. On the other hand, NPY radioiodinated at the carboxyl terminal tyrosine residue in position 36 (NPY_{36}) while still exhibiting preferential binding to LBM also exhibited specific binding to BBM. This observation raises the possibility that there are receptors on the BBM which are only readily demonstrable with NPY labelled at the carboxyl terminus and not with amino-terminal labelled NPY (NPY_1). When the putative NPY receptors were identified by cross-linking to radiolabelled NPY no receptor species unique to NPY_{36} was identified.[16] It is possible that the binding reflects contamination of BBM with LBM. However, the degree of binding observed with the carboxyl terminal labelled tracers makes this unlikely. Since BBM contain proteases, preferential degradation of NPY_1 and

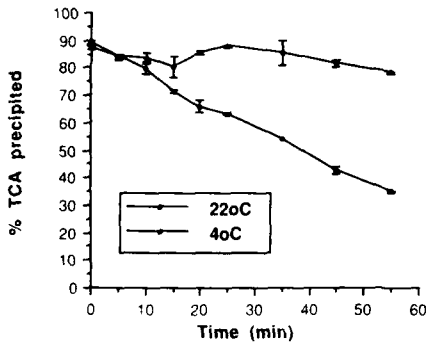


FIGURE 1. *Time and temperature dependence of Bolton-Hunter-NPY degradation by BBM.* B-H-NPY was incubated with BBM (20 μg of membrane protein) for the times noted on the abscissa. After the incubation was completed, the suspension was centrifuged at 27,500 x g for 30 min. The degree of degradation of the unbound radiolabeled NPY contained in the supernatant was assessed by 10% TCA precipitation (30 min at 4°C).

BH-NPY relative to NPY_{36} could account for the apparent differential binding of the different radioligands to BBM.

To address this question, NPY label degradation was assessed by monitoring either the fraction of radiolabelled NPY that did not precipitate with 10% TCA or the fraction which could not be rebound to LBM. In the latter experiments, Bolton-Hunter labelled NPY was incubated for 1 hr at 22°C, either in the absence of membranes or in the presence of 70 μg of either LBM or BBM. At the end of the incubation period, the radiolabelled NPY that was not bound to the membranes was recovered after centrifugation (27,500 x g for 30 min) and allowed to rebind to LBM, in the presence or absence of 1 μM unlabelled NPY. The specific binding of BH-NPY to LBM was determined as the difference in the amount of radiolabelled NPY bound in the absence or presence of excess unlabelled NPY. Compared to the control preincubation without membrane, preincubation with LBM did not alter specific binding to LBM in the second incubation (11.5% after preincubation with LBM or without membranes). In contrast, after 1 hr preincubation of BH-NPY with BBM, the subsequent specific rebinding to LBM was only 27% of control (specific binding of 3%). In a similar fashion, after an incubation with BBM, the unbound BH-NPY showed a 60% decrease in TCA precipitability (from 87% to 36%). Degradation of BH-NPY by BBM was time and temperature dependent (FIG. 1).

We next determined whether there was differential degradation of NPY_1 and NPY_{36} by BBM by examining degradation of different forms of IODO-GEN radiolabelled NPY (FIG. 2A). After 30 min incubations of label peaks at 22°C with 40 μg BBM, the percentages of unbound radiolabelled NPY that could be TCA-precipitated was 51% for peak 1 (NPY_1), 92% for peak 2 (NPY_{36}), and 90% for peak 3 ($NPY_{1\ \&\ 36}$). By comparison, the corresponding percentages for LBM were 89%, 96% and 94% respectively. The greater degradation of NPY_1 by BBM was little altered by the addition of an excess amount (1 μM) of unlabelled NPY. Similar results were obtained when the different forms of lactoperoxidase-labelled NPY (FIG. 2B) were studied. Thus, after incubation with 50 μg membranes, the fractions of lactoperoxidase-radiolabelled NPY that could be TCA-precipitated was 87% (peak 1) and 95% (peak 2) after a preincubation with LBM, and 40% and 82% after a preincubation with BBM. This finding correlates well with the 50% loss in the expected rebinding ability of NPY_1 to LBM after preincubation with BBM.

In the next series of experiments, we attempted to minimize enzymatic degradation of NPY. Even with shorter incubations at 4°C in the presence of a combination of protease inhibitors (1 mM of phenylmethylsulfonylfluoride and 0.0125 mg/ml each of pepstatin, chymostatin, antipain, leupeptin, trypsin inhibitor), degradation of NPY_1 was not fully inhibited. When degradation was partially inhibited, small increases in specific binding of

NPY_1 to BBM could be demonstrated, suggesting that degradation accounts at least in part for the lack of NPY_1 binding to BBM. However, since the conditions used to limit degradation (shorter incubations at 4°C) resulted in decreased specific binding of all labels, including NPY_{36}, we cannot assess with certainty whether the lack of NPY_1 binding to BBM was due entirely to degradation. In any case, regardless of the radiolabel used, NPY bound less well to BBM than to LBM, suggesting that degradation will not entirely account for the preferential binding of NPY to LBM. Since only the radioactivity of the radiolabeled NPY is monitored, we cannot determine if regions of the molecule other than those containing the labelled tyrosine residue undergo degradation. However, these results provide evidence that there is preferential degradation of the amino terminus of NPY by BBM.

Cross-Linking the NPY Receptor in Intestine and Brain

Intestine

Lacto-NPY_1 was cross-linked to its LBM receptor using DSS and the resulting NPY-receptor complexes analyzed on SDS-PAGE followed by radioautography (FIG. 3). The NPY-receptor complexes, which were cross-linked in the absence of the reducing agent DTT (FIG. 3; lane 3), migrated as a major radioactive band with an Mr of 52,000–59,000 and as a minor band with an Mr of 42,000–44,000 (the ranges reflect the apparent molecular weights estimated using the top and bottom of each band, and are derived from eight separate experiments). Radioactivity at the top of the lane represents aggregated material larger than 220 kDa (the size of the largest molecular weight marker, ferritin); the

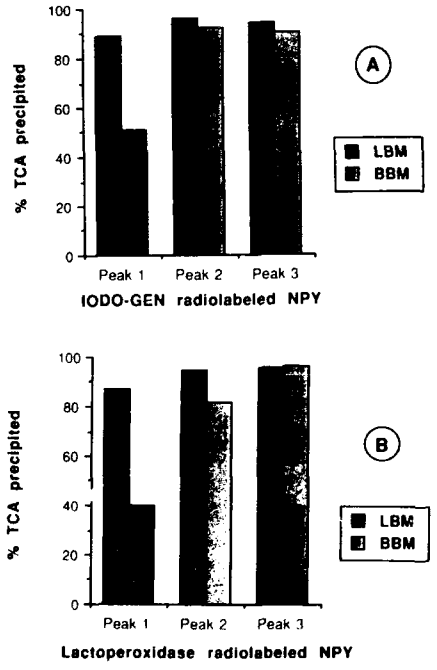
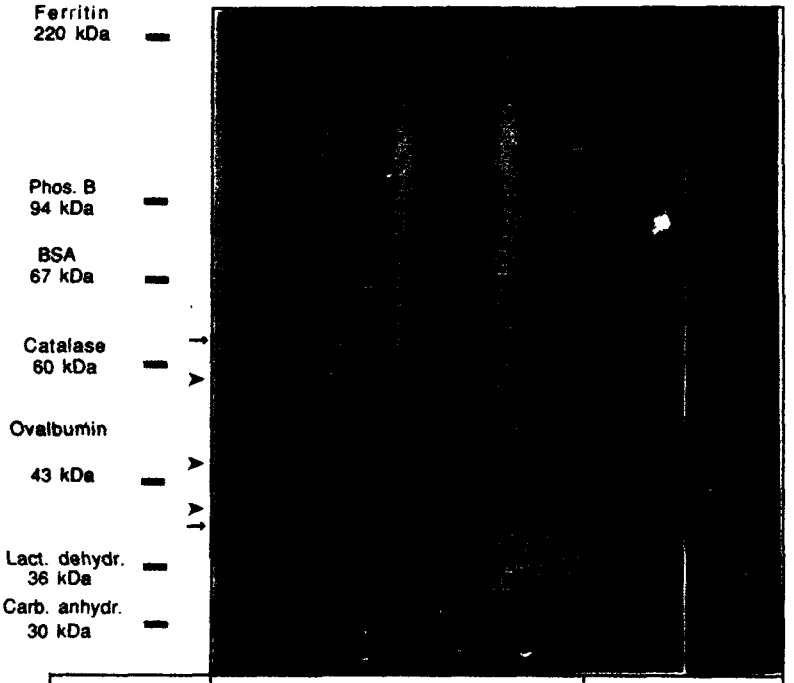


FIGURE 2. Differential degradation of radiolabeled NPY by intestinal LBM and BBM: NPY was radiolabeled with either IODO-GEN or lactoperoxidase and the different peaks obtained from HPLC purification incubated with LBM or BBM at 22°C. When the incubation was completed, the suspension was centrifuged at 27,500 x g for 30 min. The degree of degradation of the unbound radiolabeled NPY contained in the supernatant was assessed by 10% TCA precipitation (30 min at 4°C). **(A)** IODO-GEN radiolabelled NPY, incubation for 30 min with 40 μg of membrane protein; **(B)** lactoperoxidase radiolabeled NPY, incubation for 60 min with 50 μg of membrane protein.

activity at the front of the gel is thought to reflect radiolabelled NPY that was bound, but not cross-linked to the membranes, which then became dissociated during SDS treatment. Cross-linking was blocked by the addition of 10 μM cold NPY prior to the incubation with DSS (lanes 2 and 4). Incubation of the cross-linked membranes in 10 mM DTT prior to SDS-PAGE (lane 1) minimally affected the migration of the 52–59-kDA band but



Tissue	Intestinal laterobasal membrane				Brain	
DTT	+	+	-	-	+	-
Unlabeled NPY	0	10^{-5}M	0	10^{-5}M	0	0

FIGURE 3. Size difference between intestinal and brain NPY-receptor complexes. Intestinal laterobasal membranes (37.5 μg of membrane protein) or crude brain membranes (370 μg membrane protein) were incubated with trace amounts of lactoperoxidase radioiodinated NPY in the presence or absence of unlabeled 10 μM NPY. The NPY-receptor complexes were next cross-linked using 0.2 mM (intestinal) or 1 mM (brain) of DSS and analyzed subsequently by SDS 10% polyacrylamide gel electrophoresis in the presence or absence of the reducing agent DTT. The resulting radioautograph is shown. On the *left* of the radioautograph, the positions of the molecular weight standards and the bands corresponding to the intestinal (▶) or brain (→) NPY receptors are shown.

shifted the position of the 42–44 kDa species to Mr 37–39 kDa. This later change may reflect intramolecular disulfide bonding within the 42–44 kDa species or disulfide bonding between a 37–39 kDa binding subunit and a small 5 kDa subunit. Additional faint bands at Mr ~75,000–90,000 and ~110,000–120,000 were also revealed; these addi-

tional bands reflect specific binding of NPY in that they were inhibited in the presence of 1 μM unlabelled NPY.

Brain

When radioactive NPY was cross-linked to the brain receptor with DSS, two radioactive bands were seen (FIG. 3: lane 6) which migrated at approximately 62 kDa (range 60–64) and 39 kDa (range 36–39). In contrast to the intestinal receptor the addition of 10 mM DTT did not appreciably alter the migration pattern of either band (lane 5).

DISCUSSION

NPY is localized to the intrinsic nerves of the small intestine, and may be released from nerve endings to directly inhibit intestinal secretion.[8–11] Interaction between NPY and specific receptors located on the serosal side of the intestinal epithelial cell is a necessary prerequisite for this biologic action. Using a membrane preparation technique that allows fractionation of LBM free of ER-Golgi, we have demonstrated that NPY, radiolabelled with the Bolton-Hunter agent or at tyrosine 1 (with IODO-GEN and lactoperoxidase) binds exclusively to intestinal LBM.[16] This finding is consistent with the observation that NPY will only inhibit secretion when applied to the serosal surface of mucosal strips mounted in Ussing chambers. In competitive inhibition studies, unlabelled NPY inhibited B-H NPY binding with an IC_{50} of 20–50 nM. Thus the measured affinity of the intestinal receptor was not as high as the values previously described for the brain and kidney NPY receptors,[14,17–19] or for a PYY-preferring receptor described on crude intestinal membranes.[20] This 4- to 5-fold lower affinity may be partially due to the small degree of ligand degradation by laterobasal membranes. Alternatively, the low affinity could reflect greater receptor degradation during the 2-day-long intestinal membrane fractionation, which might occur despite the addition of protease inhibitors at the beginning of the procedure. However, it should be noted that this lower K_D is still within the range of the EC_{50} (10–30 nM) estimated for the biologic effects of NPY on the intestine.[8–11]

We have identified two tentative candidates for the LBM NPY receptor after cross-linking the receptor followed by analysis on SDS-PAGE under reducing conditions: a major 52–59 kDA band and a minor 37–39 kDa band. In the absence of DTT, the 37–39 kDa band migrates as a 42–44 kDa species. This finding suggests the presence of disulfide bonding within the receptor itself or between a 37–39 kDa binding subunit and a separate ~5-kDa subunit. IODO-GEN-NPY_2, IODO-GEN-NPY_3, and lacto-NPY_2 cross-linking to LBM with DSS revealed additional faint bands at 75–90 kDa and 110–120 kDa.[16] These faint bands were also seen with DSP cross-linking of lacto-NPY_1, and as such they do not appear to be specific for IODO-GEN-NPY_2 or lacto-NPY_2. Since these bands were faint and inconsistent, we have had difficulty in further analyzing their relationships to the 52–59 kDa- and 37–39/42–44 kDa species. Since both the 52–59 and 37–39 kDa species have the same affinity for NPY, we speculate that the smaller product is derived as a result of degradation from the larger species.

While Inui *et al.*[21] reported the molecular weight for the NPY/PYY receptor in the brain to be of 50 kDa, we demonstrated two candidate species for the brain NPY receptor, with Mr's of 62,000 and 39,000.[23] The slight difference in size between the intestinal 52–59 kDa species and the brain 62 kDa species was confirmed when the cross-linked NPY-receptor complexes from these two different tissues were analyzed together on the

same gel (FIG. 3). Another difference between intestine and brain is that the smaller species in the intestine [37–39/42–44 kDa] is sensitive to reducing agents while the brain 39 kDa species is not. Alternate methodologies (*e.g.*, immunologic mapping, receptor purification and amino acid analysis or receptor cloning) will be required to determine whether these slight differences reflect distinct structures, or artifactual modifications of the receptor secondary to enzymatic degradation of the larger species (75–90 kDa or 115–120 kDa).

IODO-GEN-NPY_1 and IODO-GEN-NPY_2 bound equally well to the brain NPY receptor,[14] while IODOGEN-NPY_2 bound better than IODO-GEN-NPY_1 to LBM and BBM derived from enterocytes. The binding of IODO-GEN-NPY_2 and IODO-GEN-NPY_3 coupled with the lack of binding of IODO-GEN-NPY_1 to brush border membranes is intriguing. IODO-GEN-NPY_1 is iodinated at tyrosine residue 1, IODO-GEN-NPY_2 is iodinated at tyrosine residue 36, and IODO-GEN-NPY_3 is iodinated at both tyrosine residues 1 and 36. The differential binding of the different labels to LBM and BBM could reflect two classes of NPY receptors: a class localized to LBM which can bind B-H-NPY and NPY radioiodinated at the tyrosine residue 1, and an additional class localized to BBM (and possibly LBM) which only recognizes NPY radioiodinated at position 36. However, in our cross-linking experiments, we have been unable to provide structural evidence for distinct NPY receptors which can only bind NPY radiolabelled at position 36. It is conceivable that binding of NPY to BBM reflects contamination with LBM. However, the magnitude of binding of NPY_{36} labels to BBM makes this unlikely as BBM would have to be heavily contaminated. Our studies in differential degradation of different label species by LBM and BBM suggest the presence in BBM of an enzyme(s) that cleaves the amino terminus of NPY thereby removing the label in the Tyr_1 position and the Bolton-Hunter label.

In summary, we have localized receptors for NPY to the serosal laterobasal membrane of the intestinal epithelial cell. By covalently cross-linking NPY to its receptor, we have demonstrated two main molecular species of the receptor with molecular sizes of 48–55 kDa (52–59 kDa minus molecular weight of NPY) and 33–37 kDa. While NPY iodinated at $Tyrosine_1$ is extensively degraded by BBM we have observed that NPY iodinated at $Tyrosine_{36}$ will bind to BBM.

SUMMARY

Neuropeptide Y (NPY) is widely distributed in the central and peripheral nervous systems where it serves neuromodulator and neurotransmitter functions. NPY is contained within intrinsic nerves of the small intestine and can be demonstrated to inhibit intestinal secretion when added to the serosal side of intestine mucosa mounted in Ussing chambers. When injected centrally it has potent effects on food intake, blood pressure, sexual activity and circadian rhythms. Using NPY radiolabeled with iodogen, lactoperoxidase, or the Bolton-Hunter reagent, we have localized high-affinity NPY receptors on brain membranes and on the serosal laterobasal membranes of the rat intestinal epithelial cell. We have demonstrated that enzymatic degradation may limit the ability to demonstrate NPY binding to brush border membranes. In other experiments NPY was cross-linked to its receptors in brain and intestine using disuccinimido suberate and the resulting complexes analyzed on SDS polyacrylamide gel electrophoresis followed by radioautography. We identified two main NPY receptor species in the intestine with molecular sizes of 52–59 kDa and 37–39 kDa. The 37–39 kDa species may possess a disulfide bond which gives the receptor a fixed conformation, or it may be composed of two subunits (37–39 kDa and ~5 kDa subunits). This conclusion is based on the different migration of the

smaller band in the presence of the reducing agent, dithiothreitol. The intestinal NPY receptor exhibits differences from the rat brain receptor previously characterized by us using similar techniques. The brain receptor has a molecular weight of approximately 58 kDa with a smaller species of about 35 kDa which shows no differences in migration after exposure to dithiothreitol. The localization of NPY receptors on laterobasal membranes and brain membranes is consistent with previous anatomic and physiologic findings. The different characteristics of each receptor type provides physical evidence of receptor heterogeneity. However, it is possible that the greater enzymatic degradation observed in intestinal membranes might explain the differences in receptor sizes in the two organs.

ACKNOWLEDGMENT

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Neuropeptide Y Receptors: Autoradiographic Distribution in the Brain and Structure-Activity Relationships[a]

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INTRODUCTION

Following the isolation of neuropeptide Y (NPY) and related peptides (such as PYY) by Tatemoto and colleagues,[1-3] it rapidly became evident that these substances possess multiple central and peripheral effects (see REF. 4 for recent review) including the stimulation of food intake,[5-8] the modulation of various cardiovascular parameters[9] and the inhibition of the electrically stimulated rat vas deferens.[10-12]

In the CNS, NPY-like peptide immunoreactivity is broadly distributed being especially concentrated in hypothalamic and cortical areas.[13-25] In fact, it appears that NPY is one of the most abundant peptides in the brain. On the other hand, presence of genuine PYY and/or pancreatic polypeptide (PP)—immunoreactive materials in the brain has been more controversial, although recent data suggest the existence of PYY- and PP-like substances in the brain-stem.[26-29] It is also of interest to note that NPY-like material is often colocalized with noradrenaline in caudal brain regions[20] while being present in somatostatin and/or GABA neurons in forebrain areas such as the cortex and the

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striatum.[14,21,25] This has been used as means to address the possible significance of neurotransmitters' colocalization.[30]

NPY and related peptides most likely induce their various biological effects by activating specific membrane-bound receptor sites.[31–49] Early on, we sought to characterize these putative receptor sites using membrane binding assays,[35] bioassays[50,51] and receptor autoradiography.[35] Following our original report on the qualitative autoradiographic distribution of [^{125}I]NPY binding sites in rat brain, it became evident that important mismatches existed between the discrete localization of NPY-binding sites and NPY-immunoreactive fibers and terminals.[30,52] Consequently, we explored various hypotheses which could explain these apparent discrepancies. They are briefly reviewed here. In addition, they clearly reveal that major issues remain to be resolved before we can fully address the extent of the physiological role(s) of NPY and homologues in brain and peripheral tissues.

NPY Binding Sites in Rat Brain: Autoradiographic Distribution

Following our original report on the autoradiographic distribution of [^{125}I]NPY binding sites in the rat brain,[35] various other groups have reported similar results[36,37,44,53] and we have recently published an extensive study on the *quantitative* localization of [^{125}I]NPY sites in this tissue.[54]

As shown in FIGURES 1 and 2, [^{125}I]BH-NPY binding sites are discretely distributed in the rat brain. High densities of specific labelling are especially seen in various layers and subfields of the hippocampus (oriens layer and stratum radiatum), superficial layers of the cortex, certain thalamic nuclei, lateral septum, anterior olfactory nuclei, mammillary bodies and inferior colliculus. Low to moderate levels of [^{125}I]BH-NPY binding are found in the striatum and most hypothalamic and brainstem nuclei.[54]

The presence of high quantities of [^{125}I]BH-NPY binding sites in cortical and hippocampal area correlates well with the reported behavioral and electrophysiological effects of NPY and homologues which are most likely mediated by neuronal cells located in these regions. For example, it has been shown that NPY can inhibit population spike in hippocampal slices[55,56] and facilitate learning and memory in mice.[57] However, the presence of low levels of [^{125}I]BH-NPY binding in hypothalamic and brainstem areas[58] is rather puzzling since it is well known that PP-related peptides induce multiple effects (*e.g.*, food intake,[5–8] body temperature,[4] modulation of cardiovascular parameters[9]) generally believed to be mediated by cells located in these two brain regions. It also contrasts with the presence of high amounts of NPY-like immunoreactive materials (cell bodies, fibers and terminals) found in many hypothalamic and brainstem nuclei while comparatively sparse NPY-like fibers and terminals are seen in the hippocampal formation.[13–25] These apparent mismatches have been discussed in detail elsewhere.[30,52] Possible explanations include the existence of major species differences in the localization of NPY-like immunoreactive materials and binding sites, the rat being an exception, as well as the presence of multiple classes of NPY receptors in the brain, only a given subtype being recognized under incubation conditions used in most binding experiments.

NPY Binding Sites in the Brain of Various Mammalian Species

Globally, NPY binding sites appear to be similarly distributed in rat, hamster, guinea pig, pig, monkey and human brain.[58–60] In all species studied thus far, high quantities of specific labelling are found in the hippocampal formation and various cortical laminae.[58–60] However, some differences in labelling intensities have been observed in

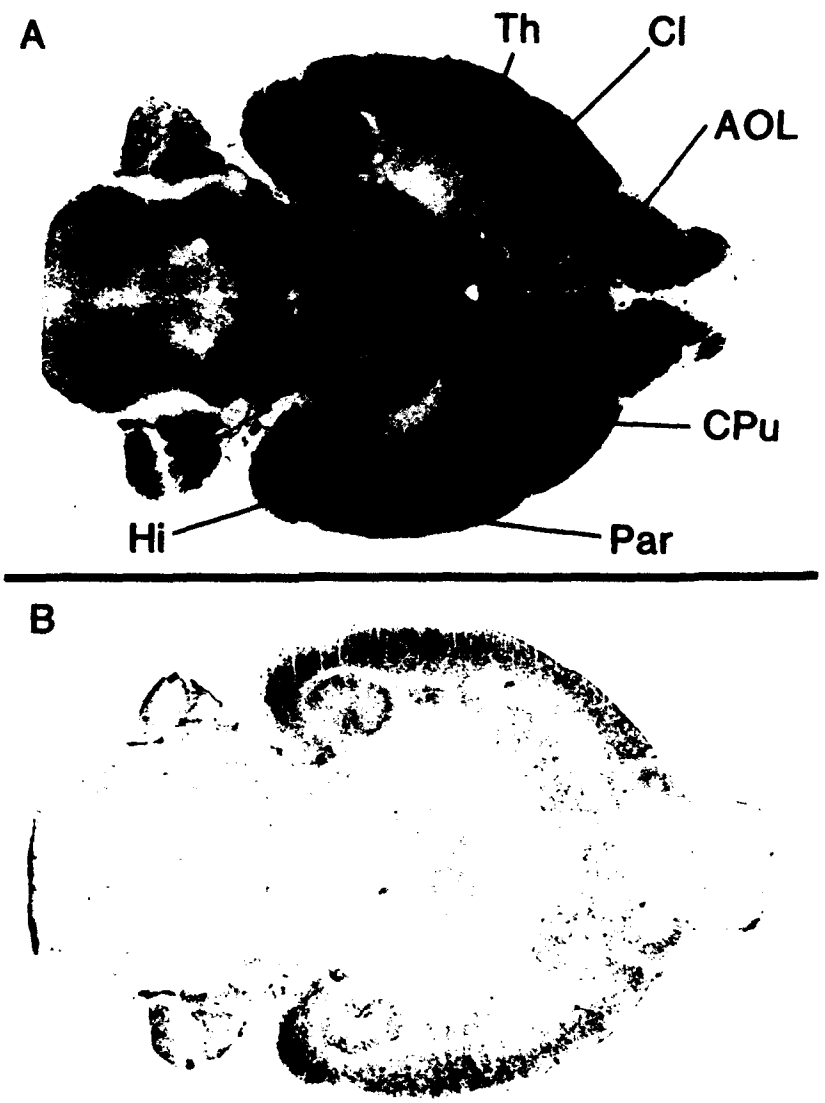


FIGURE 1. Horizontal sections of the rat brain at approximately Bregma = −7 mm depicting the overall distribution of [^{125}I]BH-NPY receptor binding sites. The total binding is shown in **(A)** while the nonspecific labelling remaining in presence of 1.0 μM NPY is shown in **(B)**. Note the high densities of sites present in cortical and hippocampal areas. Abbreviations: AOL, lateral part of the anterior olfactory nucleus; CL, claustrum; CPu, caudate-putamen; Hi, hippocampus; Par, parietal cortex and Th, thalamus. (Modified from Martel *et al.*[54])

the striatum, septum and thalamus.[58] For example, while the rat caudate-putamen contains relatively low amounts of binding, this same area is enriched in [^{125}I]BH-NPY binding sites in the guinea pig brain. Thus, possible species differences in the localization

of NPY-like materials and receptor sites can not be excluded and care must be taken when generalizing from data obtained in a given species. On the other hand, it is very unlikely that mismatches between the distribution of NPY and NPY receptors can be fully explained on the basis of phylogenic differences.

Comparative Distribution of NPY and PYY Receptor Sites in the Rat Brain

Apparent receptor mismatches could also be related to the existence of multiple receptor subtypes and/or states. Early on, Wahlestedt *et al.*[61] proposed the existence of at least two classes of NPY receptors (Y_1 and Y_2) on the basis of the differential pre- and postjunctional effects of certain C-terminal fragments in *in vitro* bioassays. Other groups have recently provided additional evidence for the existence of these two classes of NPY receptors using other assay systems,[41,44,46–48,53,62] although it now appears that this nomenclature is not fully inclusive, additional receptor subtypes being most certainly present in certain preparations (*e.g.*, PP receptors, N-terminal sensitive sites, etc.).[39,40,43,53,62–65]

We[54] and others[44] have investigated the comparative autoradiographic distribution of sites labelled by NPY and PYY radioreceptor probes in order to determine if these ligands can differentiate between receptor subtypes and/or states. This is especially relevant since it has been shown that both peptides are equally effective in inducing certain biological effects while in other tissues PYY and NPY differ in their relative potencies (see REF. 4 for details). Overall, it appears that [^{125}I]BH-NPY and [^{125}I]PYY bind to a similar class of sites in the rat brain since the autoradiographic distribution of specific labelling is similar with both probes.[44,54] For example, the labelling pattern is almost identical in areas such as the hippocampal formation, the cortex and various thalamic nuclei (FIG. 3). Autoradiograms are generally of better quality with [^{125}I]PYY, most likely because of the higher affinity and greater specificity of this radioligand for the labelled sites. Consequently, if both probes are used at identical concentrations, resulting autoradiograms may give the impression that [^{125}I]PYY differentially labels specific sites in these areas.

However, labelling profiles are most likely different in various hypothalamic and brainstem nuclei, and in the cerebellum (FIGS. 3,4).[54] While low amounts of specific labelling are observed in these regions with a NPY probe, [^{125}I]PYY apparently binds to moderate to high densities of sites in certain hypothalamic and brainstem nuclei as well as in the cerebellum (FIGS. 3,4). For example, high amounts of [^{125}I]PYY binding are seen in the lateral hypothalamus (FIG. 3), nucleus of the solitary tract (FIG. 4) and the hypoglossal nucleus (FIG. 4). This clearly demonstrates that [^{125}I]PYY can detect the presence of important quantities of NPY/PYY receptor sites in these regions that are apparently not as well recognized using NPY probes. However, it still remains to be established if these sites represent a genuine receptor subtype or merely a different state of a single class of NPY receptors. It is also likely that PP-related ligands may recognize yet another class of sites that would be almost exclusively located to the area postrema, nucleus of the solitary tract and interpeduncular nucleus.[53]

NPY Ligand Selectivity Pattern and Structure-Activity Relationships in CNS and Peripheral Tissues

Better characterization of the possible existence of NPY/PYY/PP receptor subtypes will require the development of highly selective receptor probes acting as either agonists or antagonists. We,[63–66] as well as other groups,[12,34,38,41–49,53,61,62,67,68] have begun an

extensive investigation of the structure-activity relationships of NPY receptors in various assay systems.

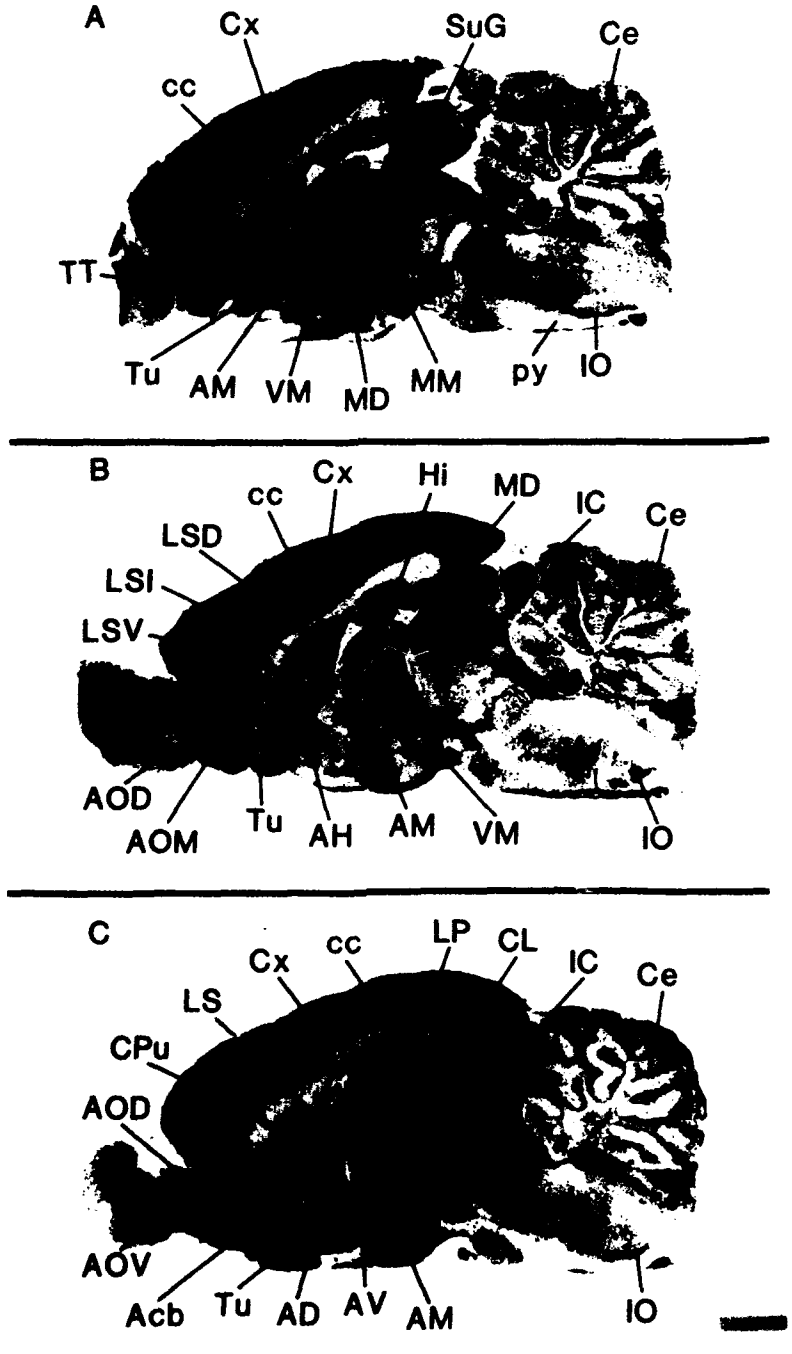
First, we focus on C- and N-terminal fragments since it had been reported that long C-terminal fragments such as $NPY_{(13-36)}$ were comparatively more potent on Y_2 than Y_1 receptor sites.[61] As shown in TABLE 1, it appears that C-terminal fragments $pNPY_{(2-36)}$, $pNPY_{(13-36)}$ and $NPY_{(18-36)}$ are able to compete for [^{3}H]NPY binding sites in rat brain membrane preparations and to mimic, with full intrinsic activity, the inhibitory effect of NPY in the electrically stimulated rat vas deferens. However, their relative potencies in these two preparations were much lower than that of NPY itself. Globally, this profile is similar, but not identical, to that of the Y_2 receptor.[61] Differences are mostly related to the lower than expected relative potency of $pNPY_{(13-36)}$, especially in the rat brain binding assay (TABLE 1).

Other fragments such as $NPY_{(25-36)}$, and N-terminal fragments $NPY_{(1-15)}$ and $pNPY_{(1-24CONH2)}$ are basically inactive in both preparations at up to a 1.0 μM concentration. This suggests that amino acid residues responsible for triggering receptor activation (intrinsic activity) are most likely located in the C-terminal half of the NPY molecule. On the other hand, amino acid residues critical for maintenance of appropriate receptor recognition (affinity) appear to be present in both the C- and N-terminal portions of the molecule as exemplified by the losses of potency/affinity observed with the removal of the tyrosine residue in position 1, and between fragments $NPY_{(18-36)}$ and $NPY_{(25-36)}$ (TABLE 1). This correlates well with the purported tertiary structure of PP-like molecules in which both N- and C-terminal residues appear to be in close proximity.[69-72]

We also obtained rather intriguing results which tend to suggest that the Y_1/Y_2 receptor classification may not be fully inclusive. For example, we observed that relatively high concentrations (μmolar range) of the N-terminal fragment $pNPY_{(1-24CONH2)}$ can potentiate the contraction of the electrically stimulated vas deferens, an effect opposite to that of NPY itself.[63] In addition, at a lower concentration which did not induce a stimulatory action, this N-terminal fragment apparently slightly antagonized the inhibitory effect of NPY.[63] This could be a useful starting point toward the development of NPY receptor antagonists. It also suggests that the N-terminal portion of the NPY molecule could in fact possess some biological properties, as reported for a variety of other peptides including opioids[73] and neurokinins.[74] Moreover, it would certainly be of interest to determine if any of these fragments can be generated *in vivo*.

A few puzzling results were also obtained with C-terminal fragments.[64,65] As shown in FIGURE 5, while the profile of activity of various C-terminal fragments in the rat colon correlates rather well with a Y_2 selectivity pattern ($pNPY \geq pNPY_{(13-36)} \geq pNPY_{(2-36)} \geq NPY_{(18-36)}$), the potency and activity of $pNPY_{(13-36)}$ in the guinea pig main bronchus is rather unique. In this preparation, this C-terminal fragment induces a much greater

FIGURE 2. Sagittal sections of the rat brain at approximately lateral −0.1 mm **(A)**, lateral 0.4 mm **(B)** and lateral 1.4 mm **(C)** showing the overall distribution of [^{125}I]BH-NPY receptor binding sites. Abbreviations used: Acb, nucleus accumbens; AD, anterodorsal thalamic nucleus; AH, anterior hypothalamic area; AM, anteromedial thalamic nucleus; AOD, anterior olfactory nucleus, dorsal part; AOM, anterior olfactory nucleus, medial part; AOV, anterior olfactory nucleus, ventral part; AV, anteroventral thalamic nucleus; cc, corpus callosum; Ce, cerebellum; CL, centro-lateral thalamic nucleus; CPu, caudate putamen; Cx, cortex; Hi, hippocampus; IC, inferior colliculus; IO, inferior olive; LP, lateral posterior thalamic nucleus; LS, lateral septum; LSD, lateral septum, dorsal part; LSI, lateral septum, intermediate part; LSV, lateral septum, ventral part; MD, mediodorsal thalamic nucleus; MM, medial mammillary nucleus; py, pyramidal tract; SuG, superficial gray layer of the superior colliculus; TT, tenia tecta; Tu, olfactory tubercule; VM, ventromedial thalamic nucleus. (Modified from Martel *et al.*[54])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maximal contraction than pNPY itself, although the threshold concentration required to produce a minimal contraction is lower for the native peptide (FIG. 5).[64] This is certainly different from the usual Y_2 receptor profile and deserves further investigation.

We also observed that $pNPY_{(2-36)}$ is more potent than pNPY itself to increase food intake following direct injection in the CNS (TABLE 2).[65] This is rather unique since this same fragment is generally less potent than the full peptide in a variety of binding and bioassay preparations (for example, see TABLES 1 and 2). Similar results have recently been reported by Magdalin and colleagues[75] and Kalra *et al.*[76] Thus, it would appear that

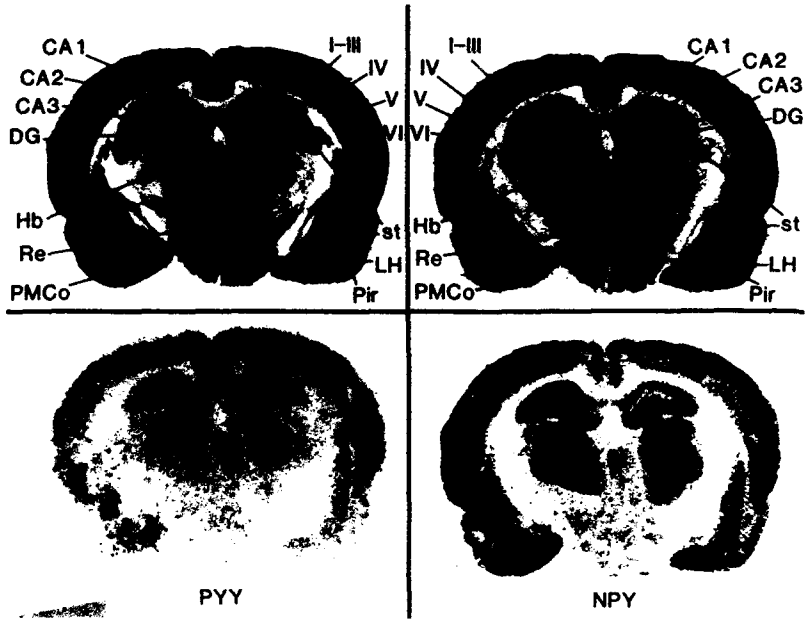

FIGURE 3. Coronal rat brain sections at approximately Bregma = −3.6 mm showing the comparative distribution of [^{125}I]PYY *(left)* and [^{125}I]BH-NPY *(right)* receptor binding sites. While both populations of sites are similarly distributed in cortical and hippocampal areas, there is an apparent abundance of [^{125}I]PYY sites in the hypothalamus. The *top panels* show total binding while the *bottom panels* reveal nonspecific labelling remaining in the presence of 1.0 μM PYY or NPY, respectively. Abbreviations: I-VI, cortical laminae I to VI; CA_1, CA_2, CA_3, subfields of the Ammon's horn of the hippocampus; DG, dentate gyrus; Hb, habenula; LH, lateral hypothalamic area; Pir, piriform cortex; PM Co, posteromedial cortical amygdaloid nucleus; Re, reuniens thalamic nucleus; and st, stria terminalis. (Modified from Martel *et al.*[54])

the tyrosine residue in position 1 is not critical for the activation of the NPY receptor subtype mediating the effect of this peptide family on food intake behavior.

Finally, on the basis of the loss of receptor activation (intrinsic activity) seen between fragments $NPY_{(18-36)}$ and $NPY_{(25-36)}$ in the vas deferens preparation, we decided to evaluate the effects of various modifications of the tyrosine doublets in positions 20 and 21.[63] As shown in TABLE 1, certain analogues seem to possess preferential affinity for either brain or vas deferens NPY receptor sites. Substitutions in position 20 ([D-Tyr^{20}] or D-Trp^{20}]hNPY) produced analogues demonstrating 4-fold higher relative potencies in the

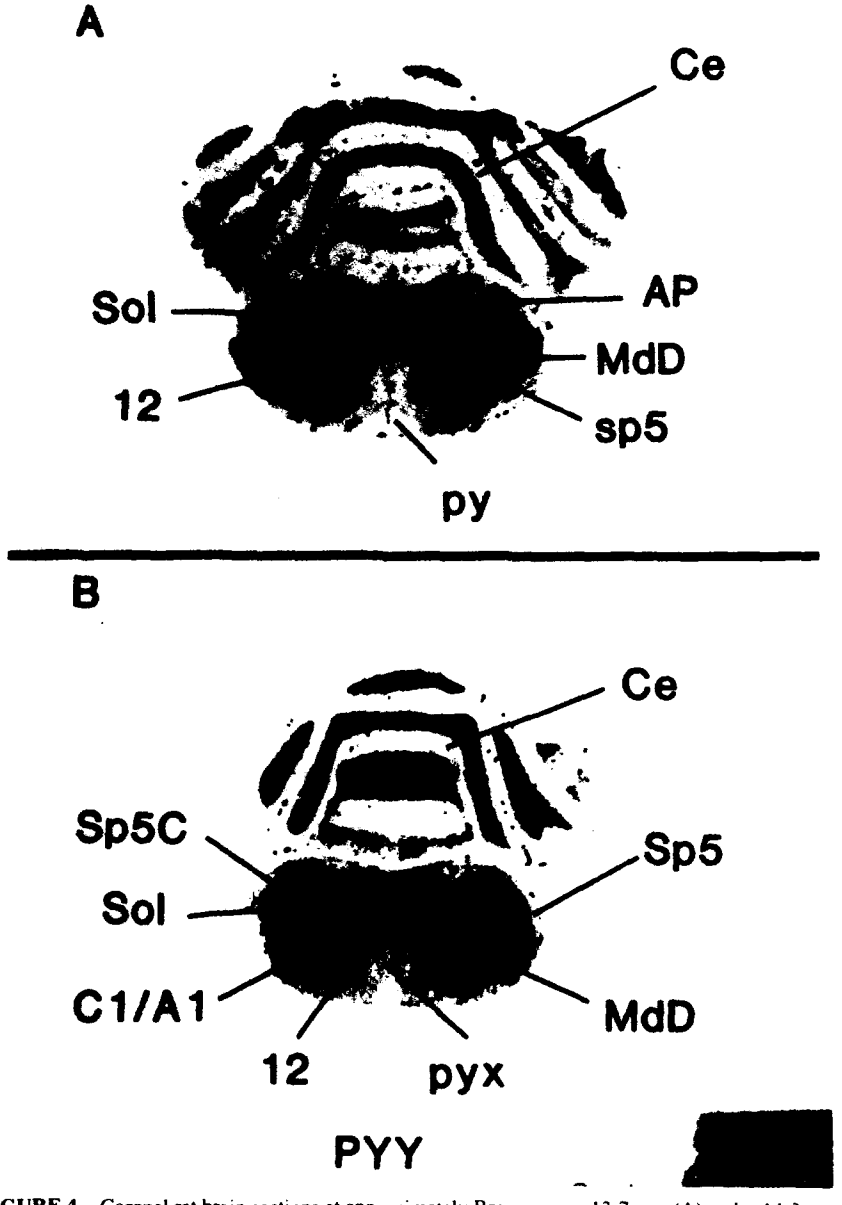

FIGURE 4. Coronal rat brain sections at approximately Bregma = −13.7 mm **(A)** and −14.3 mm **(B)** depicting the distribution of [^{125}I]PYY receptor binding sites. At these levels, labelling with [^{125}I]BH-NPY is rather low and mostly nonspecific while [^{125}I]PYY binding is highly specific both in the cerebellum and the brainstem. Abbreviations used: AP, area postrema; Ce, cerebellum; C1/A1, C1 adrenaline cells/A1 noradrenaline cells; MdD, medullary reticular nucleus, dorsal part; py, pyramidal tract; pyx, pyramidal decussation; Sol, nucleus of the solitary tract; Sp5, spinal trigeminal tract; Sp5C, spinal trigeminal nucleus, caudal part; and 12, hypoglossal nucleus. (Modified from Martel *et al.*[54])

TABLE 1. Comparative Affinities or Potencies of Various NPY Fragments and Analogues in the Rat Brain Binding Assay and the Rat Vas Deferens Bioassay[a]

Peptide	Relative Affinity (%) Rat Brain Binding	Relative Potency (%) Rat Vas Deferens
pNPY	100	100
$pNPY_{(2-36)}$	7	20
$pNPY_{(13-36)}$	0.6	6
$NPY_{(18-36)}$	0.7	7
$NPY_{(25-36)}$	<0.04	<2.0
$NPY_{(1-15)}$	<0.04	<2.0
$NPY_{(1-24CONH2)}$	<0.04	<4.0
[D-Tyr^{20}]hNPY	4.3	17
[D-Trp^{20}]hNPY	3.3	12
[Phe^{21}]hNPY	119	7.2
[Tyr-O-Me^{21}]hNPY	56	3.9

[a]Rat brain membrane preparations were prepared as described by Martel *et al.*[35,63] using [^{3}H]propionyl-pNPY (0.5 nM) as radioligand. Each fragment and analogue has been tested at concentrations ranging between 0.01 and 10,000 nM. Rat vas deferens were prepared as described by Martel *et al.*[63] Each fragment and analogue has been tested at concentrations ranging between 0.5 and 1000 nM. Relative affinities and potencies as compared to pNPY taken as 100%.

rat vas deferens while modifications in position 21 ([Phe^{21} or Try-O-Me^{21}]hNPY) generated analogues having relative selectivity for the rat brain NPY binding sites (TABLE 1). This could eventually lead to the development of selective CNS vs PNS analogues, in addition to revealing subtle differences in the structural requirements of NPY receptors present in the rat brain and vas deferens preparations.

In summary, future studies should aim at the development of highly selective agonists and antagonists for each putative receptor subtype. Such tools are most critical in order to fully examine the respective physiological significance of a given receptor class. The recent development by Fuhlendorff *et al.*[47] of [Leu^{31}, Pro^{34}]NPY as a selective Y_1 receptor agonist is certainly most promising in that regard.

Possible Interaction between NPY, Sigma and Phencyclidine Receptor Sites

Recently, Roman and colleagues[77] reported that NPY and PYY were able to compete, with nanomolar affinity, for sigma and phencyclidine (PCP) receptor sites. This is interesting and suggests that PP-related peptides may act as endogenous ligands of the sigma and/or PCP receptors. It is now well established that PCP binding sites are associated with the N-methyl-D-aspartate (NMDA) receptors[78] while the functional significance of sigma sites is still mostly unknown.[78] Thus, further investigations of the possible interactions between NPY/PYY-like peptides, and sigma and PCP receptor sites are certainly warranted. This could eventually lead to the development of new pharmacological tools, based on either the PCP or sigma pharmacophore, for the characterization of NPY receptor subtypes.

CONCLUSIONS

In summary, NPY receptor sites are widely, but discretely, distributed in mammalian brains with high densities being located in various cortical and hippocampal areas. A

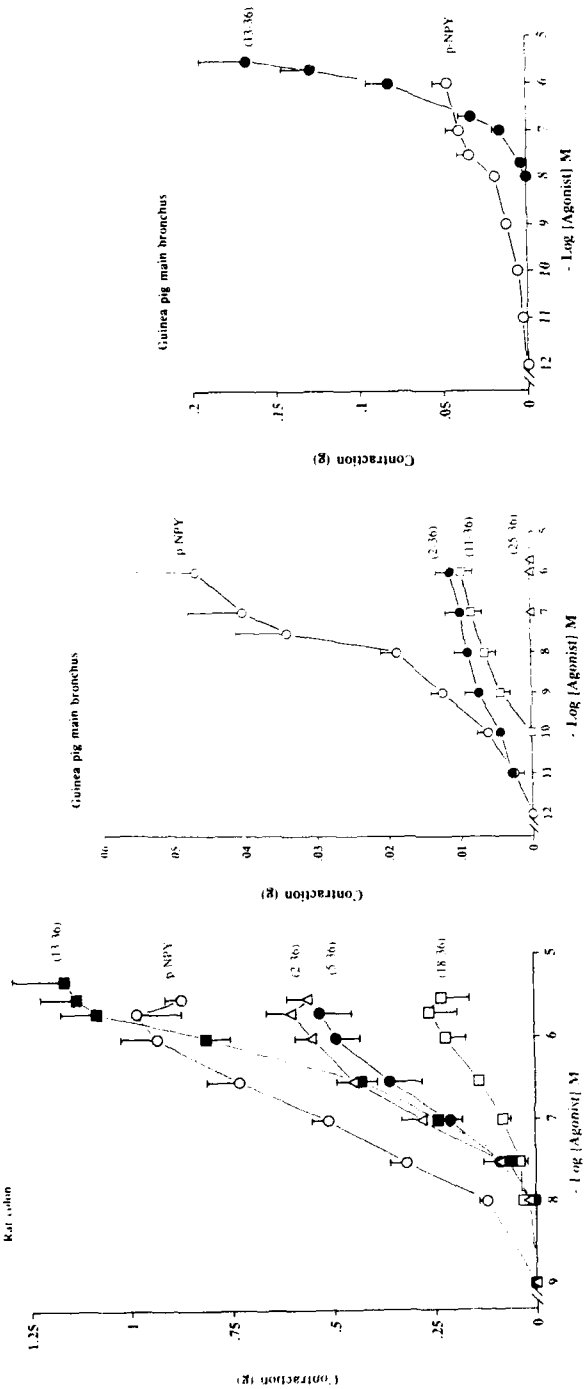

FIGURE 5. Comparative concentration-response curves of porcine-NPY (pNPY) and related C-terminal fragments in inducing contractions in the isolated rat colon *(left)* and the isolated guinea pig main bronchus (*center* and *right*). In both preparations, p-NPY is more potent than C-terminal fragments $NPY_{(2-36)}$, $NPY_{(13-36)}$, $NPY_{(11-36)}$, $NPY_{(18-36)}$ and $NPY_{(25-36)}$. Fragment $NPY_{(13-36)}$ is as effective as pNPY itself to induce a maximal contraction in the rat colon although higher minimal concentrations are required to induce an effect *(left)*. In the guinea pig main bronchus, pNPY induced a contraction at lower concentrations than $NPY_{(13-36)}$. However, the fragment is much more effective than the native peptide in inducing a maximal effect *(right)*. (Modified from Cadieux *et al.*[64])

TABLE 2. Relative Affinities of Various NPY Fragments in Inducing Certain Behavioral Effects of NPY Following ICV Injections[a]

Peptide	Decreased Motor Activity (%)	Increased Food Intake (%)	Hypothermia (%)
pNPY	100	100	100
$pNPY_{(2-36)}$	30	130	20
$pNPY_{(13-36)}$	20	4	<0.1
$NPY_{(25-36)}$	<0.1	<0.1	<0.1
$NPY_{(1-24CONH2)}$	<0.1	<0.1	<0.1

[a]For experimental details, see REFERENCE 65. Relative potencies as compared to pNPY taken as 100%.

ditionally, NPY/PYY receptor subtypes most likely exist in CNS and peripheral tissues. This hypothesis is based on the differential labelling of hypothalamic and brainstem nuclei by NPY and PYY receptor probes, and on the respective ligand selectivity profile and the differential order of potency of agonists found in various bioassay systems. However, the development of potent and selective receptor antagonists is awaited in order to provide more definite evidence for the existence of NPY receptor subtypes.

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Pancreatic Polypeptide and Peptide YY Gene Expression[a]

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INTRODUCTION

Pancreatic polypeptide, peptide YY, and neuropeptide Y are members of a family of regulatory polypeptides. Pancreatic polypeptide is one of the four major hormones synthesized in pancreatic islets. The primary biological effect of pancreatic polypeptide is inhibition of pancreatic exocrine secretion. Pancreatic polypeptide secretion is stimulated by a variety of nutritive and/or neurogenic factors, most of which appear to be mediated by cholinergic pathways. Peptide YY has been localized primarily to ileum and colon.[1-5] Peptide YY immunoreactivity has also been identified in pancreas, jejunum, duodenum, and gastric antrum.[2,5] The physiological actions of peptide YY include inhibition of gastric emptying, intestinal motility, pancreatic exocrine secretion, and secretions of acid and pepsin from the stomach.[1,2,6,7] Peptide YY is released in response to the presence of fat in the intestine.[8,9] In addition to the direct effect of fat on peptide YY release,[10] postprandial secretion of peptide YY appears to be stimulated by signals originating in the foregut.[11] Present evidence suggests that cholecystokinin is a candidate foregut factor which stimulates peptide YY release.[11]

We previously isolated cDNAs encoding the precursors of human pancreatic polypeptide[12] and rat peptide YY.[13] The amino acid sequences of the precursor polypeptides predicted from cDNAs reveal a conserved precursor structure shared by all three members of this peptide family.[12-15] Each precursor consists of a signal peptide 28 or 29 amino acids in length, a 36-amino-acid mature hormone followed by a Gly-Lys-Arg sequence, and a C-terminal peptide consisting of approximately 30 residues (FIG. 1). A unique tyrosine amide is present at the C-terminus of the mature polypeptides. The marked similarities in precursor structures for pancreatic polypeptide, peptide YY, and neuropeptide Y, are consistent with the hypothesis that each of these peptides arose by duplication of a common ancestral gene.

To study the structure and expression of the genes encoding pancreatic polypeptide and peptide YY, we isolated the genes encoding rat peptide YY and both human and rat pancreatic polypeptide. Here we show that the structure of the rat peptide YY gene shares

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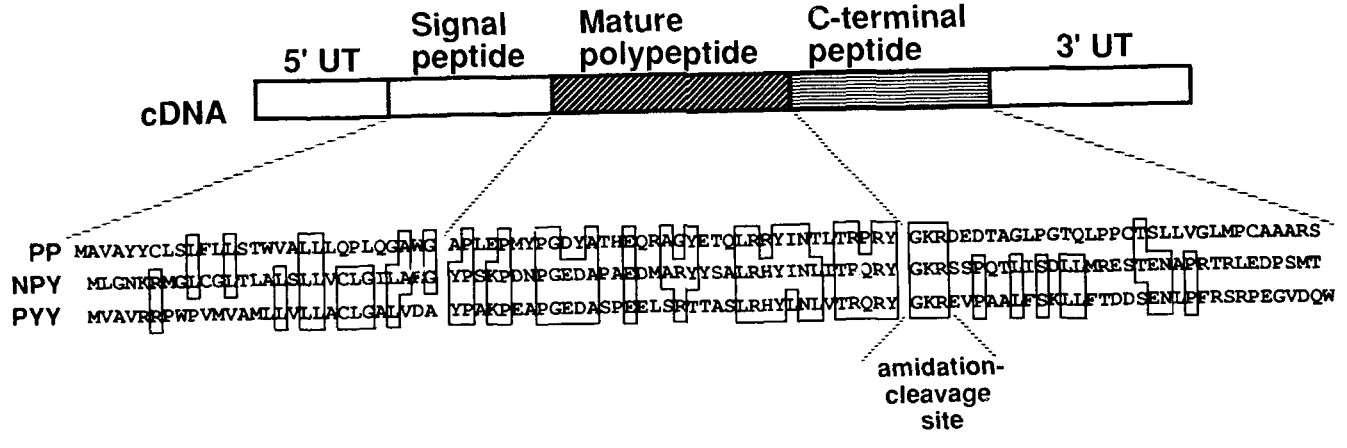

FIGURE 1. Amino acid sequences of the rat neuropeptide Y, pancreatic polypeptide, and peptide YY precursors predicted from the cDNA. Conserved amino acid sequences are enclosed in *boxes*. Abbreviations are: NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY.

a common structural organization with the genes encoding neuropeptide Y and pancreatic polypeptide. We also show that the peptide YY gene is developmentally regulated and that the expression of this gene is regulated by protein kinase-dependent intracellular signalling pathways.

MATERIALS AND METHODS

Isolation of the Gene Encoding Rat Peptide YY

The gene encoding rat peptide YY was identified from a rat genomic library constructed in bacteriophage λ using an oligonucleotide probe corresponding to the cDNA sequence. Positive clones were plaque-purified by sequential low density plating, and bacteriophage DNA was isolated by the plate-lysate method. A single 4.5 kb Kpn I restriction fragment was identified by the peptide YY probe in Southern blot hybridizations, and subcloned into a plasmid vector. DNA sequencing was carried out by the chain termination method using T_7 DNA polymerase.[16] Computer-assisted nucleotide sequence analysis was performed as described by Devereux.[17]

Determination of Peptide YY Gene Expression in the Developing Rat

Peptide YY gene expression in ileum and colon of developing rats was investigated by Northern blot hybridization assays. Immediately following removal, tissues were frozen in liquid nitrogen and stored at −70°C. Total cellular RNA was prepared using RNAzol™ (Cinna/Biotex Inc., Friendswood, TX) according to the manufacturer's instructions. To assure that equal amounts of RNA were loaded onto gels for Northern blotting experiments, RNA was quantitated by the absorbance at 260 nm, and by the UV fluorescence intensity of the ribosomal RNA subunits on nondenaturing agarose gels stained with ethidium bromide. Following electrophoresis on denaturing agarose gels, RNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) by capillary blotting. Hybridizations were carried out under conditions of high stringency (65°C, 50% formamide) using antisense RNA probes for either rat peptide YY[13] or rat glucagon.[18] To assure that equal amounts of mRNA were loaded onto each lane, the blots were reprobed for β-actin.

Promoter Activity of the Peptide YY Gene in Transient Expression Assays

To study the transcriptional regulation of the rat peptide YY gene, we introduced reporter gene constructions into HeLa cells for transient expression assays. For these experiments, fusion genes were constructed by inserting 280, 242, 183 or 127 bp of peptide YY 5′ flanking sequence and 33 bp of exon 1 upstream from the reporter gene for the bacterial enzyme, chloramphenicol acetyl transferase (CAT). The DNA fragments containing 280, 242, or 183 bp of 5′ flanking sequence were isolated by restriction endonuclease digestion of the genomic subclone. The DNA fragment containing 127 bp of 5′ flanking sequence was amplified from the peptide YY genomic subclone with Thermus aquatis (Taq) DNA polymerase I (Cetus-Perkin Elmer, Norwalk, CT) using the polymerase chain reaction.

HeLa TK-cells were plated at a density of 10^6 cells per 100 mm plates in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with the plasmid DNA by calcium phosphate precipitation.[19] After a 24 h transfection period, the medium was

replaced with medium containing 0.5% fetal bovine serum. Thirty-six h later, cells were treated with combinations of forskolin and 3-isobutyl-1-methyl xanthine (IBMX) to increase intracellular cyclic AMP, and the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to activate protein kinase C. Cells were harvested 16 h later, and extracts were prepared by freeze-thawing and sonication. Extracts containing either 50 or 100 μg of protein were assayed for CAT activity as described by Gorman,[20] and the results were normalized to 100 μg of protein.

RESULTS AND DISCUSSION

Structure of the Rat Peptide YY Gene

The transcription unit of the rat peptide YY gene spans approximately 1.2 kb and consists of four exons separated by three introns. Each exon of the rat peptide YY gene defines a functional domain of its mRNA, as do the corresponding exons of the pancreatic polypeptide and neuropeptide Y genes[21–25] (FIG 2). Exon 1 of the rat peptide YY gene contains the 5′ untranslated region of the mRNA. The second exon of the rat peptide YY gene begins at the translational initiation site and encodes the signal peptide and all but the C-terminal tyrosine of the mature polypeptide. Exon 3 encodes the C-terminal tyrosine of peptide YY, a Gly-Lys-Arg amidation-cleavage sequence, and most of the C-terminal extension peptide (FIG. 2). Like the third exons of the rat and human neuropeptide Y and human pancreatic polypeptide genes,[21–23] the splice-donor site for the third intron of the rat peptide YY gene occurs at an arginine residue in the C-terminal peptide. Although this arginine residue has been suggested to serve as a cleavage site in the pancreatic polypeptide precursor of several species,[26] it is not known whether the peptide YY precursor is cleaved at this site. The final exon of the rat peptide YY gene encodes the remainder of the C-terminal peptide and the 3′ untranslated region of the mRNA.

The amino acid sequence homology among neuropeptide Y, pancreatic polypeptide, and peptide YY as well as the conserved structural organization of the respective polypeptide precursors and genes, suggests that each member of this family arose from an ancient gene duplication event. The amino acid sequences of peptide YY and neuropeptide Y are more homologous to each other than to pancreatic polypeptide, suggesting that the former two peptides are more closely related to each other than to pancreatic polypeptide. Analogous to the amino acid sequences, the nucleotide sequences of the exons of peptide YY are more homologous to the respective exons of neuropeptide Y, than to those of pancreatic polypeptide. This observation supports the hypothesis that peptide YY has diverged more recently from neuropeptide Y than from pancreatic polypeptide.

The human neuropeptide Y and pancreatic polypeptide genes have been localized to chromosomes 7 and 17, respectively, suggesting that this gene family originated from a tandem duplication of an ancient linkage group followed by chromosomal translocation.[27] Several other gene families including *erb* proto-oncogenes, type 1 collagen, actin, band 3 membrane protein, and protein kinases, segregate similarly to pancreatic polypeptide and neuropeptide Y. Genes encoding *erbA*, *erbB-B2*, Hox 1, alpha 1 chain type 1 collagen, erythroid band 3, and protein kinase C localize on chromosome 17 along with pancreatic polypeptide. The *erbB*, Hox2, alpha 2 chain type 1 collagen, β-actin, non-erythroid band 3, and protein kinase A localize to chromosome 7 with neuropeptide Y. The protein kinase A and β-actin genes are themselves duplicated on chromosome 7 leading to the speculation that the peptide YY gene may localize to chromosome 7 as well.

Divergence of the C-terminal Peptide of the Rat Pancreatic Polypeptide Gene

The structural organization of the rat pancreatic polypeptide gene differs somewhat from the genes encoding other members of the pancreatic polypeptide gene family. The

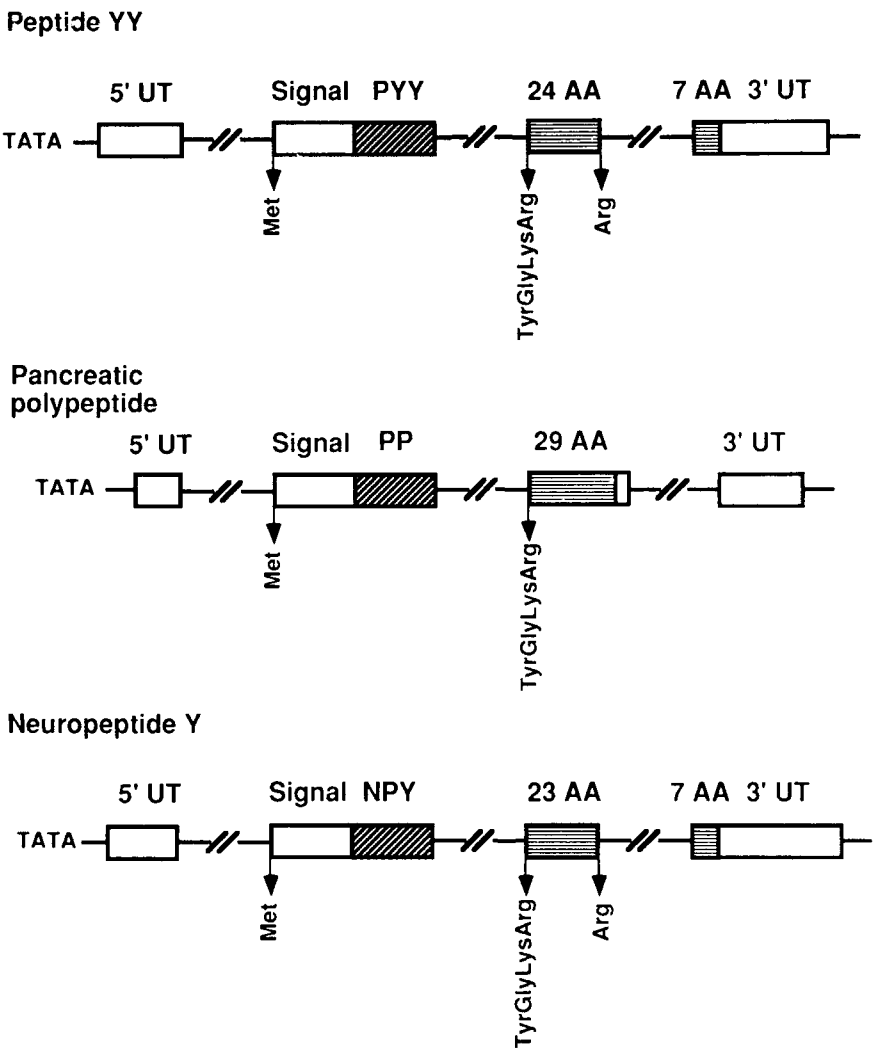

FIGURE 2. Structural organization of the rat genes in the pancreatic polypeptide family. Exons are indicated as *boxes*. Protein coding regions are *shaded*. Flanking and intron sequences are shown as *solid lines*. Abbreviations are: TATA, Goldberg-Hogness promotor; 5′ UT and 3′ UT, 5′ and 3′ untranslated regions of the mRNA; signal, signal peptide; AA, amino acid.

third exon of the rat pancreatic polypeptide gene encodes the entire C-terminal peptide and a portion of the 3′ untranslated region of the mRNA,[24,25] whereas exon 3 of the other members of this gene family, including human pancreatic polypeptide, encode only a portion of the C-terminal peptide and none of the 3′ untranslated region.[21–23] Examination of the amino acid sequences encoded by exon 3 of the rat pancreatic polypeptide gene

reveals little similarity to the corresponding sequences encoded by the human gene. To examine the molecular basis for the divergence of the C-terminal peptide of the rat pancreatic polypeptide gene, we and others[24,25] have compared the amino acid and nucleotide sequence of this gene with that of the human pancreatic polypeptide gene.

Examination of the nucleotide sequence of the rat and human pancreatic polypeptide genes reveal two mechanisms which may explain the divergence of the C-terminal peptide of propancreatic polypeptide. The first mechanism is the use of an alternative splice-donor site at the 3′ end of rat exon 3. As a result of recruitment of an alternate downstream splice-donor site, the third exon of the rat gene is 42 bases longer than its human counterpart (FIG. 3A). These additional 42 bases, which encode 11 amino acids and a translational stop codon, share identity with the corresponding sequences of human intron 3. An unused splice-donor site is present in rat exon 3 at a position corresponding to the human splice-donor site (FIG. 3A). A second mechanism contributing to the C-terminal peptide divergence is a single insertion/deletion at the 5′ end of exon 3 which produces a translational frameshift (FIG. 3A). The divergence of the C-terminal peptide sequence, therefore, appears to result from a translational frameshift mutation and the utilization of an alternate downstream splice-donor site in rat exon 3.

The nucleotide sequences at the 5′ end of exon 4 of the rat and human genes are highly conserved, despite the observation that only the human sequence is translated (FIG. 3B). Exon 4 in the rat gene encodes only the 3′ untranslated region of the pancreatic polypeptide mRNA while exon 4 of the human gene encodes 7 amino acids of the C-terminal peptide, and the 3′ untranslated region. The nucleotide sequence encoding the heptapeptide in the human gene share 19 of 25 bases with the corresponding nucleotide sequence in the rat gene. This observation suggests that prior to the existence of the frameshift mutation and the utilization of an alternate splice-donor site in rat exon 3, the 5′ region of rat exon 4 was at one time translated. The differences in the C-terminal domains of the rat and human pancreatic polypeptide precursors reflect less strict evolutionary constraints than those imposed upon the N-terminal domains of the precursors suggesting that the C-terminal peptides either have different functions or represent a region of the precursor which is unnecessary for biological activity. Despite the striking differences in the C-terminal peptide sequences, the similarities in nucleotide sequence of rat and human exons suggest that these exons arose from common ancestral exons rather than by substitution of an unrelated exon.

Ontogeny of Rat Peptide YY Gene Expression

Peptide YY immunoreactivity has been co-localized with enteroglucagon-like immunoreactivity in endocrine cells of ileum and colon in developing and adult animals.[3,4] To determine whether peptide YY and glucagon gene expression are regulated similarly during development, we compared the time-course of peptide YY gene expression to that of glucagon in the intestinal tract of fetal and postnatal rats by Northern blot hybridization assays. In the ileum, both peptide YY and glucagon mRNA were first detected on day 19 of gestation and rose to adult levels one day later (FIG. 4). In colon, peptide YY and glucagon mRNA were first detected two days earlier than in the ileum (fetal day 17), and similarly rose to adult levels by day 20 *in utero* (not shown). Peptide YY immunoreactivity in rats has previously been identified in intestinal endocrine cells at day 19 of gestation,[28] whereas enteroglucagon-like immunoreactivity has been detected in intestine as early as fetal day 16.[29] Thus, the time-courses of peptide YY and glucagon gene expression are similar in developing intestine suggesting that these two genes are developmentally regulated by a common mechanism.

We have shown that the peptide YY gene is expressed at adult levels in intestine

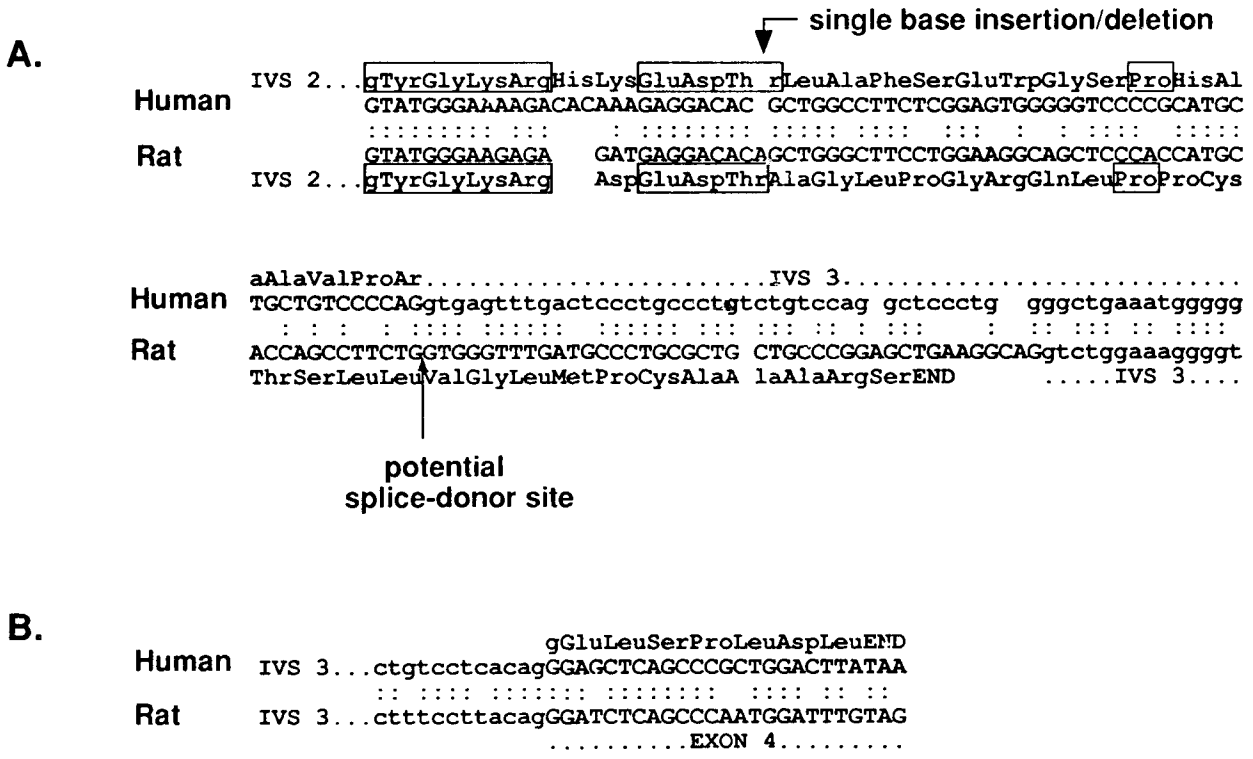

FIGURE 3. Nucleotide sequence alignment in the C-terminal peptide regions of the rat and human pancreatic polypeptide genes **(A)** Alignment between exon 3, exon 3/intron 3 junction of the rat and human pancreatic polypeptide genes. **(B)** Alignment of the intron 3/exon 4 splice junction and 5′ sequences of exon 4 of the rat and human pancreatic polypeptide genes. Exons are indicated as *capital letters* and introns are represented as *lower case letters*. Amino acid sequences of translated regions are indicated *above* or *below* the neuropeptide sequence. Identical amino acids are enclosed in *boxes*. *Colons* denote identical neuropeptide sequences. IVS, intervening sequence.

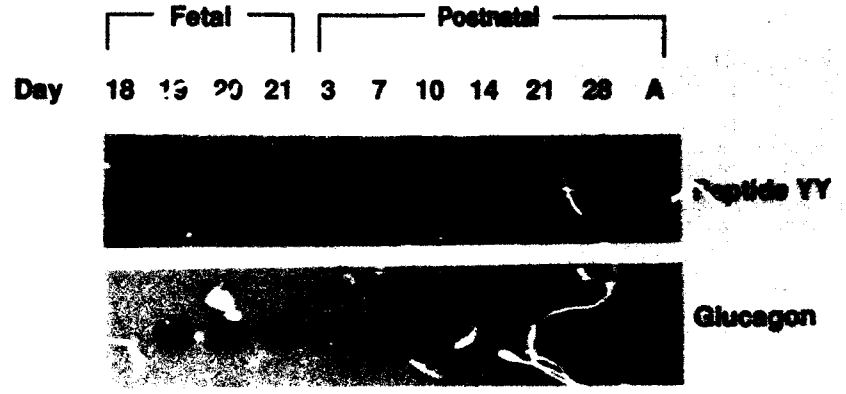

FIGURE 4. Northern blot analysis of peptide YY and glucagon mRNA in rat ileum during neonatal development. Approximately 15 μg of total RNA were loaded onto each lane. The autoradiograms represent a Northern blot hybridized first with an antisense RNA probe for peptide YY, and then with an antisense RNA probe for glucagon, at high stringency (65°C, 50% formamide). The numbers *above* each lane indicate the day of fetal or postnatal development. A, adult.

before birth antedating the putative nutritional and endocrine stimuli for peptide YY release in adult animals. Increased secretion of both peptide YY and glucagon have been associated with experimental models of intestinal epithelial cell proliferation.[30,31] The expression of the peptide YY and glucagon genes in fetal animals suggests a potential trophic role for these polypeptides during fetal development.

Regulation of Rat Peptide YY Gene Expression

Examination of the 5′ flanking sequence of the rat peptide YY gene reveals several sequences similar to cis-acting elements which are known to respond to specific intracellular signalling pathways. A potential AP-1 binding site was localized 144 bp upstream from the transcriptional initiation site, and several potential AP-2 binding sequences were identified within 130 bp of the transcriptional initiation site (FIG. 5). To determine whether these potential regulatory elements are functionally active in the rat peptide YY gene, we have carried out transient expression assays with fusion genes containing between 280 and 127 bp of 5′ flanking sequence fused to the structural gene for the bacterial enzyme CAT. The fusion genes were introduced into HeLa cells by calcium phosphate precipitation. For all fusion genes, CAT activity was induced 3- to 5-fold following treatment with the phorbol ester TPA (FIG. 5), suggesting that the sequences necessary for this induction are localized to within 127 bp of the transcriptional initiation site.

To further characterize the regulatory properties of the peptide YY 5′ flanking sequence, we introduced the chimeric construction containing 127 bp of 5′ flanking sequence into HeLa cells, and treated the cells from forskolin or IBMX to raise intracellular cAMP levels (FIG. 6). Treatment with forskolin or IBMX alone resulted in an approximate 3-fold induction in CAT activity, an induction similar to that of TPA alone. Treatment with TPA and forskolin produced a 6-fold induction in CAT activity, whereas treatment with TPA and IBMX resulted in a 7-fold induction in CAT activity. Treatment with TPA, forskolin, and IBMX resulted in a 21-fold induction in CAT activity, exceeding even the

response of the cAMP responsive element-containing 5′ flanking sequence of the vasoactive intestinal peptide gene to forskolin and IBMX[32] (FIG. 6). These data indicate that agents which raise intracellular cAMP act in association with phorbol esters to induce peptide YY gene transcription. Furthermore, the sequences necessary for this induction are also localized to within 127 bp of the transcriptional initiation site.

Our findings indicate that the potential AP-1 site at −144 is not necessary for TPA-mediated induction of peptide YY gene expression. TPA is a biologically stable analog of the second messenger diacylglycerol which activates protein kinase C. Binding of the AP-1 transactivator complex to the sequence, TGAGTCAG, is one established mechanism for transcriptional activation by phorbol esters. The potential AP-1 binding site in the peptide YY gene (AAAGTCAG) differs by two bases with the previously reported 8-base consensus sequence.[33,34] These two bases may therefore be necessary for an AP-1 site to be functionally active.

Four C-rich domains containing sequences which closely resemble transcription factor AP-2 binding sites[35,36] have been identified within 130 bp of the transcription initiation site (FIG. 5). AP-2 represents an inducible transcription factor that, like AP-1, is involved in the process of signal transduction. AP-2 may mediate transcriptional activation by phorbol esters and cAMP,[35,38] and may therefore alter the pattern of gene expression within a cell in response to two distinct signal transduction pathways. The pattern of

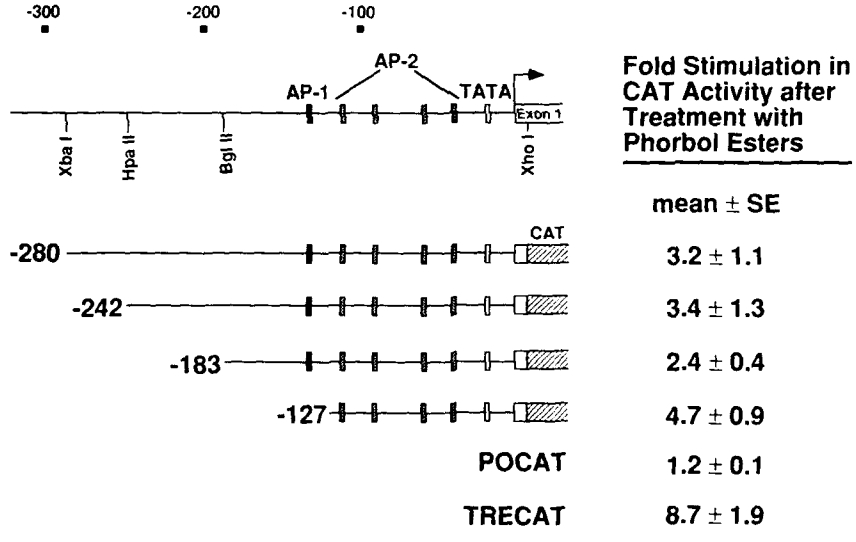

FIGURE 5. Localization of the phorbol ester responsive sequences in the rat peptide YY gene. The *solid lines* represent 5′ flanking sequence. Potential regulatory elements and exon 1 are indicated in *boxes*. Restriction sites used in the construction of chimeric peptide YY-chloramphenicol acetyl transferase (CAT) fusion genes are indicated. HeLa cells transfected with plasmid constructions containing from 280 to 127 bp of peptide YY 5′ flanking sequence were treated with TPA at a concentration of 50 nM for 16 h. A promoterless CAT construction (POCAT) and the plasmic TRECAT, which contains an active AP-1 site upstream from the enhancerless RSV promoter, were used as negative and positive controls, respectively. Cell extracts containing 50 to 100 μg protein were assayed for CAT activity. Results are expressed as the fold increase in CAT activity over untreated cells. The data are represented as mean + SE of at least three independent transfection experiments. Abbreviations are: AP, activator protein; TATA, Goldberg-Hogness promoter.

induction of CAT activity in Hela cells transfected with fusion gene containing 127 bp of peptide YY 5′ flanking sequence following treatment with TPA, forskolin, and IBMX are consistent with transactivation by AP-2.

DNA binding sites for AP-2 in enhancer regions of many genes are often found in close proximity to binding sites for other enhancer elements, suggesting that AP-2 regulates gene transcription in association with other regulatory elements. For example, in the proenkephalin gene, an AP-2 element acts synergistically with two adjacent cAMP

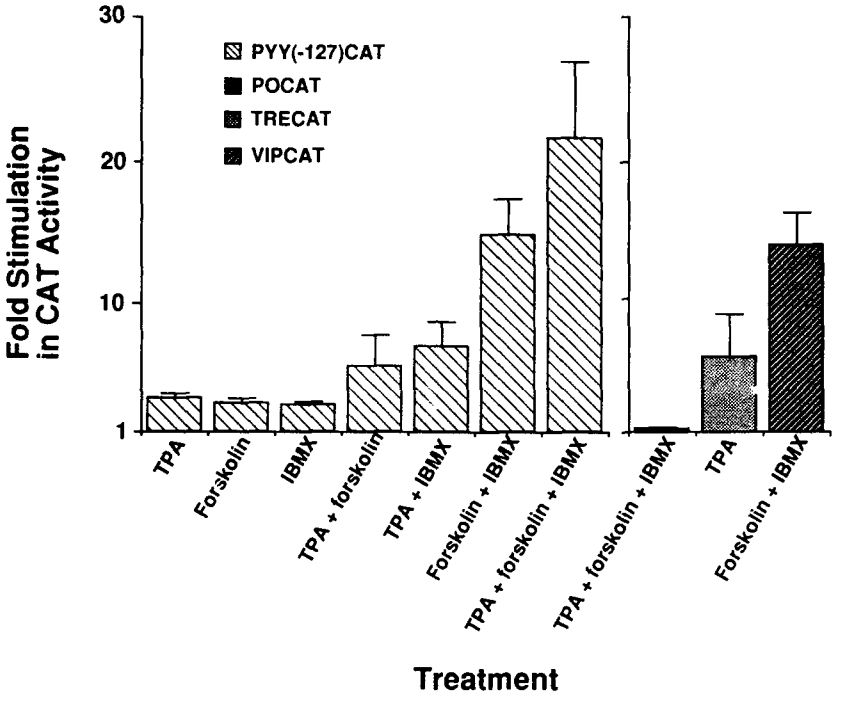

FIGURE 6. Transcriptional activation of peptide YY-CAT fusion genes by cAMP and phorbol esters. Hela cells were transfected with a plasmic construction containing 127 bp of peptide YY 5′ flanking sequence [PYY(−127)CAT]. Cells were then treated with one or combinations of the following for 16 h: TPA (50 nM), forskolin (10 μM), and IBMX (0.5 mM). POCAT and TRECAT were used as negative and positive controls as described in FIGURE 5. A CAT construction containing the vasoactive intestinal peptide cAMP responsive element (VIPCAT) was also included in these experiments for comparison. The results were determined as described in FIGURE 5. The data are represented as mean + SE of at least five independent transfection experiments.

responsive elements to produce maximal responsiveness to cAMP, although the AP-2 element alone is not sufficient for transcriptional activation (38). In the first 127 bp of peptide YY 5′ flanking sequence, no other known cis-acting DNA regulatory elements were identified, suggesting that for peptide YY, AP-2 may act alone in activating gene transcription. However, it is possible that as yet unknown enhancer elements act alone or in association with AP-2 to induce peptide YY gene transcription.

Cholecystokinin has been proposed as a candidate endocrine signal from the foregut

which stimulates the release of peptide YY.[11] Intravenous administration of cholecystokinin octapeptide (CCK-8) in dogs stimulates release of peptide YY in a dose-dependent manner.[11] Cholecystokinin may also play a role in the regulation of peptide YY gene transcription. Cholecystokinin appears to exert its effects on target tissues by increasing levels of the intracellular messengers, IP3 and 1,2-diacylglycerol, leading to mobilization of intracellular calcium and activation of protein kinase C.[39,40] In pancreatic acinar cell preparations, CCK-8 has been shown to stimulate the activity of adenylate cyclase in addition to its effects on protein kinase C.[39,41] The presence of potential cis-acting regulatory elements in the peptide YY gene which respond to elevated cAMP and activation of protein kinase C, such as AP-2 like elements, is consistent with a potential role for cholecystokinin in the transcriptional regulation of the peptide YY gene.

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Molecular Structure of Neuropeptide Y[a]

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INTRODUCTION

The actions of any neuropeptide will depend on a number of factors but will ultimately depend on the ability of the peptide released from the neuronal storage compartment to interact with its receptor. Molecular structure at all levels is likely to influence the activity of peptidergic systems. Thus, synaptic stored concentrations will be determined by the regulatory elements in nucleotide sequences flanking those of the exons that encode the peptide, the stability of the mRNA and the rate of processing of the primary translated product to the active form of the neuropeptide. Finally, the peptide must be presented in its appropriate tertiary structure to its receptors. To gain this information for neuropeptide Y (NPY) a combined approach of molecular cloning of the gene and cDNA that encode rat NPY, radioimmunoassay of the translated products and computer-aided modelling of the peptide has been used to determine the key factors involved in the regulation of the presentation of this neuropeptide to its receptors.

Structure of the Gene and cDNA That Encodes Rat NPY

A double stranded ^{32}P labelled oligonucleotide comprising 60 basepairs was used to screen a rat genomic library (from Dr. James Bonner) that had been constructed in bacteriophage Charon 4A as a partial Hae III digest. A total of 200,000 plaques were screened by hybridisation and the four positives obtained were subcloned into the cloning vector, Gemini. Subsequent analysis revealed that two clones were incomplete representations of the gene whereas two contained all the regions of the cDNA. The largest insert was 14 kb in size and the cDNA is represented in this clone by 4 exons. Exon 1 encodes the 5′ untranslated region and the first ATG, exon 2 encodes the signal peptide and the majority of NPY, exon 3 encodes the terminal tyrosine of NPY, the three amino acids required for post-translational processing and the first 23 amino acids of the flanking peptide (C flanking peptide of NPY:CPON) and exon 4 encodes the last 7 amino acids of CPON and the 3′ untranslated region. The overall structure is shown in FIGURE 1 and the precise sites of the introns are indicated by arrows on the cDNA sequence on FIGURE 2.

The same oligonucleotide probe was used to screen a rat hypothalamic cDNA library constructed in bacteriophage λgt11 (R.H. Goodman, Tufts University School of Medicine, Boston). A total number of 100,000 plaques were screened by hybridisation and at least 8 independent recombinant bacteriophages hybridised to the probe.[1] Sequence analysis of one of these inserts revealed that it comprised 559 nucleotides (FIG. 2) comprising 5′ untranslated region (nucleotides 1–68); open reading frame (nucleotides 69–365) and 3′ untranslated sequence (nucleotides 366–end) and terminated in 20 adenosyl residues preceded by a typical polyadenylation motif (AATAAA). At the start of the open reading

[a]Supported by the Wellcome Trust.

frame, there are two contiguous methionine residues. In the genomic sequence, these two methionines are separated by the first intron, as the nucleotide sequences for the first lie at the end of the first exon, whereas those for the second lie at the start of the second exon. It is unclear as to which of these two methionines is the start of translation. Assuming the first of these methionines is used, the open reading frame encodes a 98-amino-acid precursor with a signal sequence of 29 amino acids followed directly by NPY (36 amino acids); 3 amino acids necessary for post-translational processing and 30 amino acids for the flanking peptide, CPON. Remarkable homology is observed between rat[1] and human[2] NPY cDNAs at both the nucleotide level and deduced amino acid sequences. Thus, the overall homology between the two cDNAs is in the order of 60%, rising to 85% when comparing nucleotide sequences within the open reading frame. The rat sequence contains two adjacent initiation codons whereas the human sequence has only one. The deduced precursor sequences are very similar for the two species (FIG. 3). Rat and human NPY are predicted to be identical and these differ from the original porcine isolate by only one amino acid[3] at position 17 (methionine-human and rat, leucine-porcine and bovine[4]). This level of sequence conservation across species is remarkable and has been suggested to indicate conservation of structure for important functional considerations. However, cDNA analysis reveals, in addition, that between the two species the flanking peptides (CPON) are highly homologous. There are only two very conservative substitutions between rat and human CPON, in that, firstly, an alanine in the rat is substituted by a

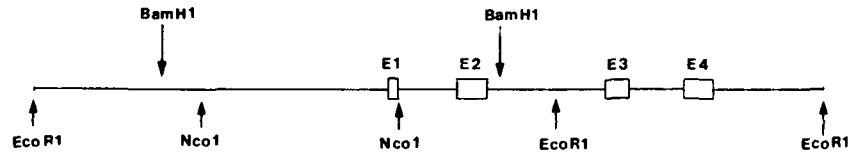

FIGURE 1. Schematic representation of the largest rat genomic fragment containing the NPY structure. The restriction enzymes sites are marked as *arrows* and the 4 exons depicted as *boxes*.

valine in the human sequence and secondly, a serine residue in the rat by an alanine in human (FIG. 3). This degree of conservation is not observed between species for another member of this family of peptides, namely pancreatic polypeptide (PP) and may suggest a potential role for CPON. The flanking peptide has been demonstrated within tissues[5] and plasma[6] by radioimmunoassay and using electron microscopy CPON-immunoreactivity has been shown to be co-stored in granules with NPY-immunoreactivity. However, in many sites where pharmacological activity of NPY has been demonstrated, application of CPON is without effect.

The overall genomic structure of NPY is similar between the two species and the exon-intron boundaries are identical in site to those for the related peptides, PP[7] and peptide YY[8] (PYY). It seems likely that the genes for these peptides have arisen as a result of duplication. Interestingly, the third intron of PP accurately predicts a further post-translational modification of the precursor, in that the flanking peptide is processed to yield two products. Although NPY possesses an intron at an equivalent position in the genomic sequence, no further processing of CPON has yet been demonstrated.

Structure of NPY

NPY is a member of the PP family of peptides. One of these, avian PP (APP) has been crystalised previously and its three-dimensional structure solved by X-ray crystallography

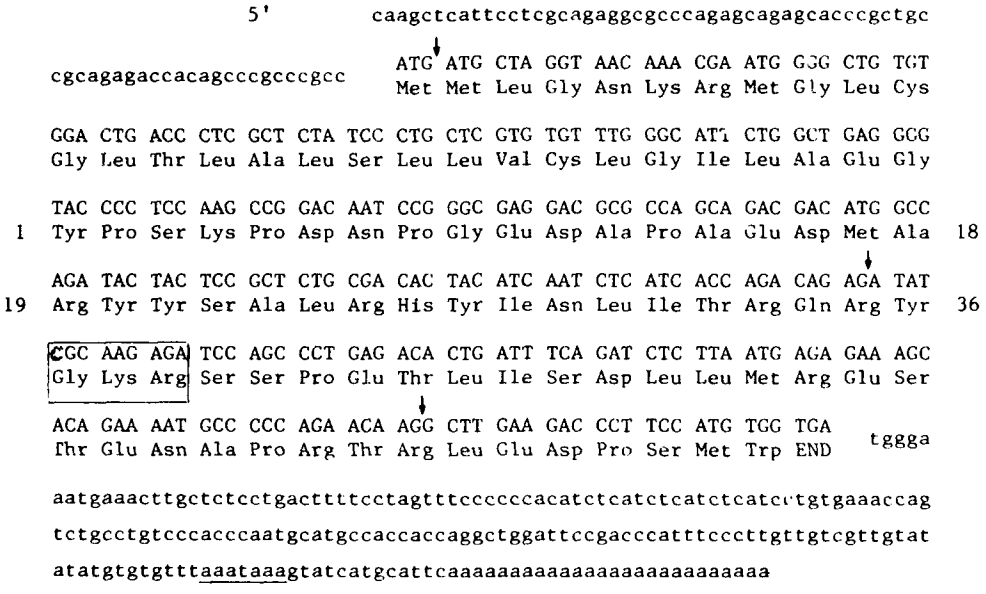

FIGURE 2. Complete nucleotide sequence of the cDNA that encodes rat neuropeptide Y. The sequences of the untranslated regions are shown in *small letters*, whereas the nucleotide sequence of the open reading frame is shown in *capitals* with the deduced amino acid sequence *below* the triplets. The N terminal tyrosine is labelled 1 and the C terminal tyrosine labelled 36. The nucleotides encoding the three amino acids for posttranslational processing are *boxed*. The polydenylation motif is *underlined*. The precise sites of the introns deduced from the genomic sequence are shown as *arrows*.

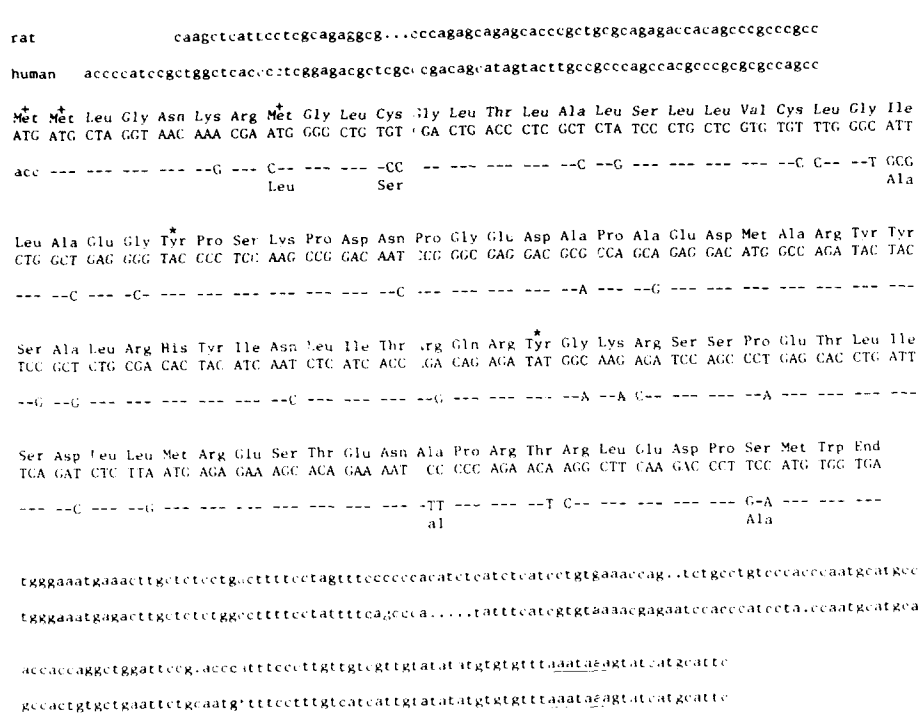

FIGURE 3. Comparison of the cDNAs encoding rat and human NPY. The rat sequences are shown *above* the human for each line. The untranslated regions are shown complete and are in *small letters*. For the open reading frame of the rat sequence, the nucleotide triplets are shown in *capitals* and the deduced amino acids are written *above* the triplets. In order to emphasize the similarity, only the nucleotides of the human sequence that differ from the rat are shown, whereas a *dash* indicates an identical nucleotide in the human sequence. Only deduced amino acids of the human that differ from that for the rat are shown on the figure and these are depicted *below* the nucleotide sequences. The N terminal and C terminal tyrosines of rat NPY are *starred*. The *dots* in the untranslated sequences represent gaps to allow alignment. The polyadenylation motifs for rat and human are *underlined*.

at high resolution.[9] Overall, only about half the amino acids are conserved between APP and NPY but closer comparison reveals that residues at certain key sites are retained between the two peptides. Based on these, it was possible to use the known crystal structure of APP to construct, by computer, a model for the structure of NPY. The model that emerged from this study indicates that the molecule has a compact and extensive tertiary structure (FIG. 4). The residues of the N terminus form a left handed polyproline-II-like helix and are brought, by a tight β turn, into spatial proximity to an α helix structure formed by residues 14 to 32. There are extensive hydrophobic interactions between the residues on the inner surfaces of these helices that stabilise the structure via intramolecular contracts. The residues of APP most important for maintaining these helix-helix contacts are either conserved in NPY or replaced by chemically homologous side chains (FIG. 5). The net result is that the modelled structure of NPY is very stable and, by this mechanism, the N and C termini of NPY are brought into spatial proximity. The model begins to explain some of the structure-function data obtained from pharmacological preparations. At least, two receptor subtypes have been proposed for NPY based on fragment activity.[10] Thus, the Y1 receptor requires the presence of intact N and C termini, whereas the Y2 receptor recognises a truncated version of NPY, 13–36 amide (*i.e.*, the C terminal polar arm and α helix). The Y1 receptor appears predominantly at postsynaptic sites in the sympathetic nervous system, whereas the Y2 receptor appears at presynaptic sites.

The requirement of the Y1 type of receptor for the whole NPY molecule for activity is best explained by the model, as the N and C terminal tyrosines are brought into spatial proximity and its seems likely that it is this face that is presented to this receptor. Perhaps the best test of the model is the maintenance of biological activity in molecules constructed on the basis of the predicted structure. Thus, a synthetic version of NPY where the truncated N (residues 1–4) and C (residues 25–36) portions were chemically linked was found to possess pressor activity.[11]

Regulation of Expression of NPY

NPY demonstrates remarkable specificity in its expression within tissue types. Apart from rodent megakaryocytes,[12] NPY-immunoreactivity is confined to cells derived from the neural crest. Indeed all three members of this family of peptides demonstrate remarkable tissue specificity, in that, just as NPY is confined to neural tissue, PP is expressed only in the pancreatic islets and PYY is present in the exocrine pancreas and in endocrine cells of the lower gastrointestinal tract.

Studies using the NPY cRNA probe to examine the distribution of NPY mRNA, in general, have demonstrated similar results to those obtained for the NPY peptide as measured by radioimmunoassay. However, in at least two instances mRNAs hybridising specifically to the labelled NPY cRNA have been found in unexpected sites, namely, rat spleen[13] and heart.[13,14] In addition, various pharmacological manipulations have shown discordant effects on the mRNA levels compared to peptide concentrations. NPY-immunoreactivity in the rat spleen is present in postganglionic sympathetic nerve terminals. Thus, the finding of high concentrations NPY mRNA in RNA extracted from these tissues was surprising and the mRNA levels were unaffected by chemical sympathetic ablation (unpublished data). Similarly, NPY-immunoreactivity within the heart is considered predominantly to be present in sympathetic nerve terminals, although small numbers of weakly positive NPY-immunoreactive ganglion cells have been demonstrated in the atria.[15,16] Specific mRNA hybridising to labelled NPY cRNA have been demonstrated in RNA extracted from all regions of the rodent heart[14] (FIG. 6) and again sympathetic ablation had no effect on the concentrations of this mRNA species despite loss of all

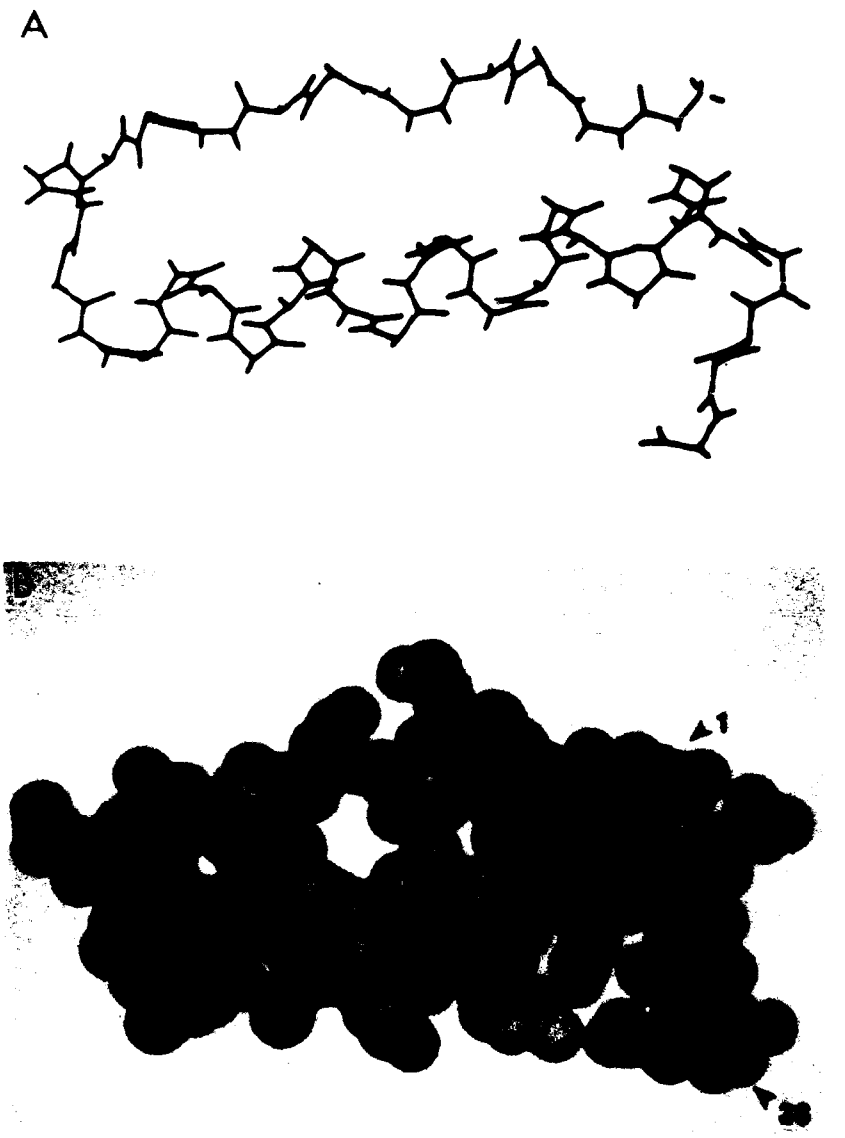

FIGURE 4. Computer-constructed NPY model. **(A)** Polypeptide chain backbone showing the polyproline-II-like helix and C terminal helix connected by a tight turn. **(B)** Same orientation as (A) but space filling representation of the NPY model. (From Allen *et al.*[1] Reprinted by permission from the National Academy of Sciences.)

measurable immunoreactivity from the same heart tissue.[14] It is difficult to explain the presence of NPY mRNA in these sites and its apparant dissociation from the peptide measurements, but this data suggests that there are situations where the mRNA is not translated to yield NPY.

A useful model system to investigate regulation of NPY gene expression in the context of neuronal differentiation is the PC 12 cell line.[17] This cell line was cloned from a rat adrenal phaeochromocytoma and the cells resemble immature chromaffin cells. These cells have been used as a model of differentiation. Dexamethasone causes the cells to become chromaffin-like, whereas nerve growth factor (NGF) halts mitosis and causes the cells to extend neurites, features of differentiation into sympathetic neurone-like cells.

PC 12 cells contain an mRNA species reactive to the NPY cRNA probe that is identical in electrophoretic size and hybridisation to the NPY mRNA in rat brain,[18] although immense variation in the amount of NPY mRNA has been observed between PC

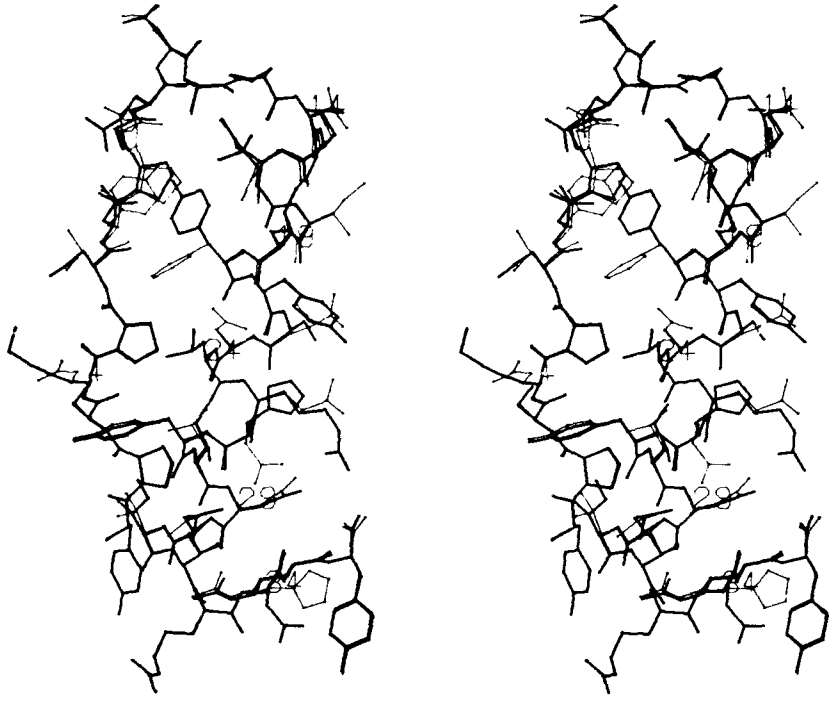

FIGURE 5. Stereoscopic view of the APP crystallographic structure (*light lines*) and the NPY model (*heavy lines*). (From Allen *et al.*[1] Reprinted by permission from the National Academy of Sciences.)

12 cells obtained from different laboratories (unpublished data). The mRNA in these cells is translated as NPY-immunoreactivity has been demonstrated within PC 12 cells.[19] By gel chromatography, the precursor appears to be processed very inefficiently. The majority (approx. 70%) of immunoreactivity elutes from size exclusion columns as a large molecular weight precursor, whereas only 30% elutes in the position of NPY itself. NPY mRNA and peptide concentrations are increased by exposure of the cells to NGF in the culture medium. An increase in NPY mRNA was observed within 12 hours (data not shown) and the response appeared to be maximal at 3 days[19] (FIG. 7). Withdrawal of NGF from the culture medium resulted in a slow return of NPY mRNA towards the control levels.

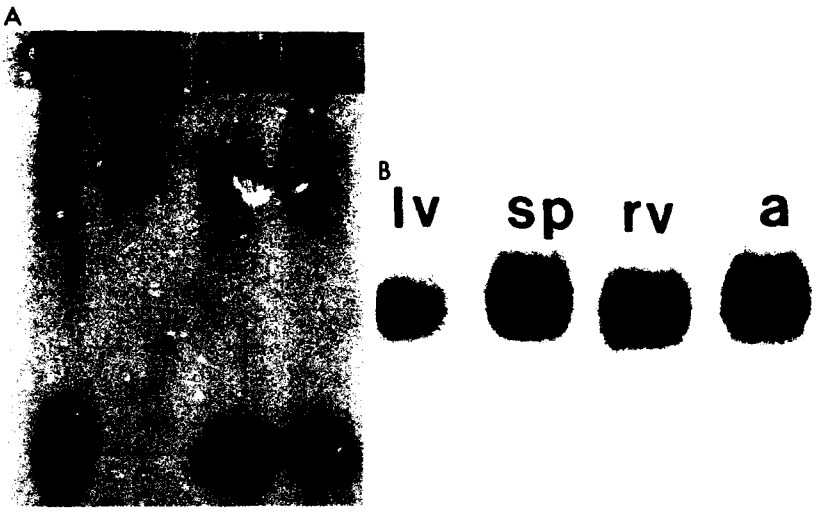

FIGURE 6. Northern blot analysis of RNA from rat heart, probed with NPY cRNA ^{32}P labelled probe under high stringency. **(A)** RNA extracted from rat brain olfactory bulb (OB) and whole heart from two animals, H1 and H2. **(B)** RNA extracted from regional dissection of heart in atria (a), left ventricle (lv), right ventricle (rv) and interventricular septum (sp).

Although the level of transcription is an important feature in the regulation of stored concentrations of NPY, it appears that in some tissues a limiting factor may be the rate of processing of the precursor to the mature peptide. The predicted precursor for NPY consists of 98 amino acids. Cleavage of the signal peptide should yield "pro-NPY" consisting of 69 amino acids (NPY: 36 amino acids, the three amino acids necessary for post-translational processing and CPON: 30 amino acids). A number of antisera have been raised to NPY and CPON. Of these YN22 recognises the N terminal part of NPY, YN10 is directed to the extreme C terminal part of NPY (it requires the C terminal amide residue for full recognition) and JN8 was raised to CPON (FIG. 8). Precursor sequences should not be recognised by YN10 but are predicted to be recognised by YN22 and JN8. Gel

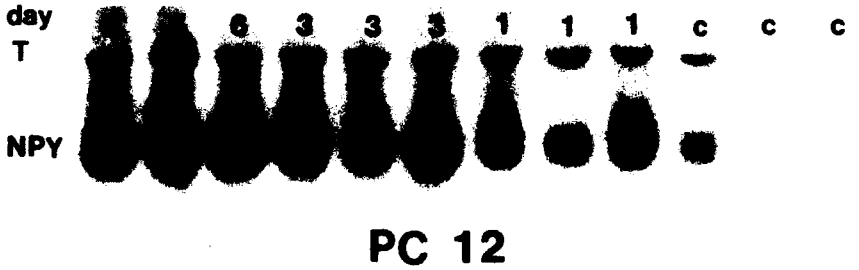

FIGURE 7. Northern blot analysis of RNA from PC 12 cells using NPY cRNA ^{32}P labelled probe and an end labelled oligonucleotide to rat tubulin (T). RNA was extracted from control cells (c) and cells treated with NGF for 1 day (1), 3 days (3) and 6 days (6).

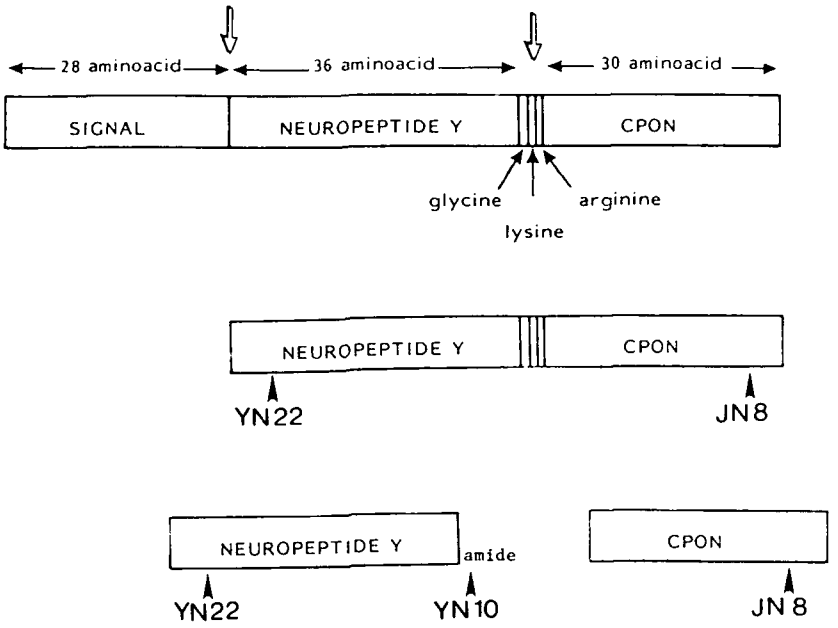

FIGURE 8. Schematic of the precursor of human NPY with an indication of the recognition sites of the three antisera YN22, YN10 & JN8. The translated product consists of a 28-amino-acid signal peptide, NPY (36 amino acids). Cleavage of the signal sequence would yield a precursor (pro NPY) that is recognised by the antisera YN22 and JN8. Processing of the precursor will generate the amidated version of NPY that is recognised by the antiserum YN10.

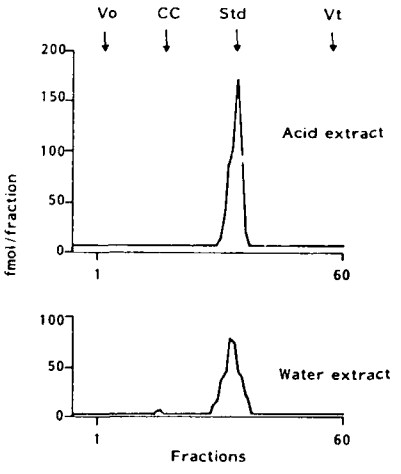

FIGURE 9. Gel permeation chromatography of acid and water extracts of rat brain. The extracts were assayed using the YN22 antiserum. All the immunoreactivity eluted from the column in the position of the NPY standard apart from a minute portion (less than 1%) that was measurable eluting from the column in the expected position of the precursor.

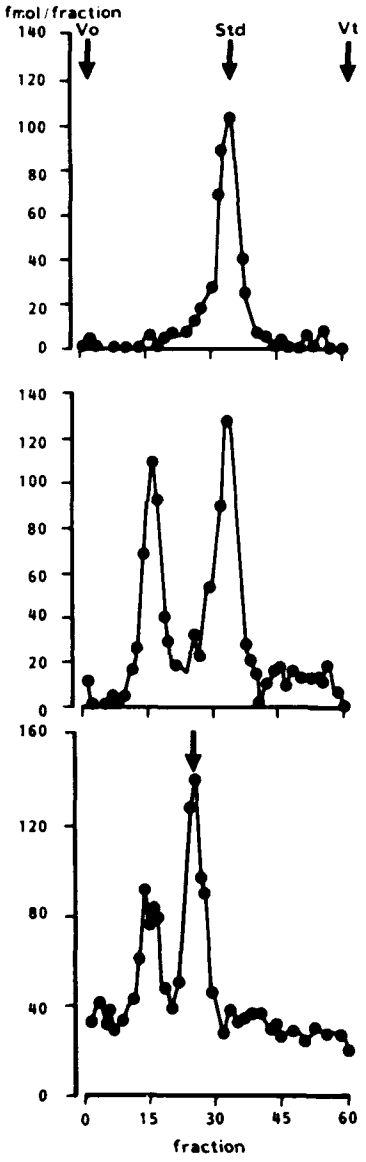

FIGURE 10. Gel permeation chromatography of extract of rat adrenal. The *upper panel* shows the profile obtained using the YN10 antiserum, where all the immunoreactivity is in the form of the mature peptide. The *middle panel* demonstrates the profile obtained by using the YN22 antiserum and the *lower panel* that for the JN8 antiserum. About 50% of the immunoreactivity elutes from the column in the position of the NPY and CPON standards respectively. However, both NPY and CPON immunoreactivity were measured in fractions that eluted earlier from the column as assessed using the two antisera YN22 & JN8 and represent the precursor form of NPY. (Modified from REFERENCE 20.)

permeation chromatography followed by assay of the fractions with the various three antisera revealed that in most tissues, the only products recognised in extracts were those of the mature peptides, NPY and CPON and no large molecular weight or small fragments were detected. This was surprisingly the case for extracts of human phaeochromocytoma tissues.[6] However, in extracts of rat superior cervical ganglia and rat adrenal tissue, significant quantities of immunoreactivity eluted from gel permeation columns in the

position of a large molecular weight form.[20] The immunoreactivity in these fractions was detectable by the two unrelated antisera, YN22 and JN8, whereas the antiserum YN10 failed to recognise any immunoreactivity in these fractions. About 25% of total immunoreactivity as assessed by both YN22 and JN8 of the rat superior cervical ganglion extracts was of the precursor form compared to mature peptide. In the rat adrenal, about 50% of the total immunoreactivity was in the form of "pro-NPY." Thus, the efficiency of precursor processing varies considerably among tissues. The pattern observed is very similar to that noted previously for the enkephalins, where the majority of enkephalin

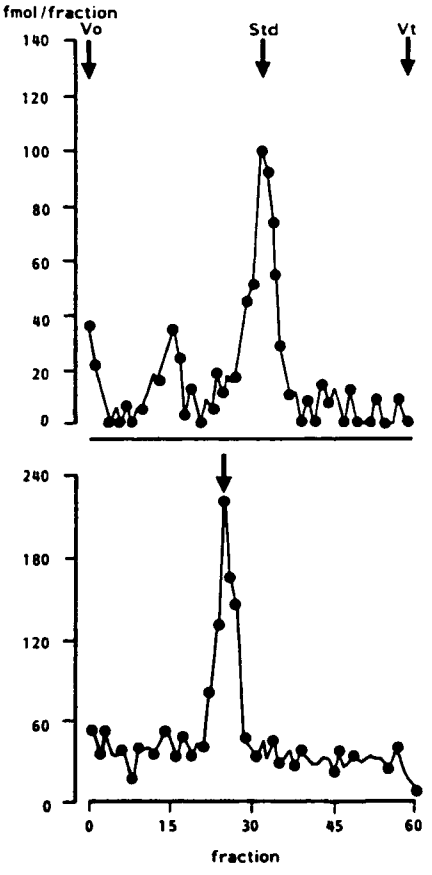

FIGURE 11. Gel permeation chromatography of the extract of adrenals collected from rats 4 hours after reserpine treatment. The *upper panel* shows the profile of immunoreactivity for the YN22 antiserum whereas the *bottom panel* is that for the JN8 antiserum. In contrast to FIGURE 10, immunoreactivity is only detectable as the mature peptide and no distinct large molecule weight precursor was observed.

immunoreactivity in the brain is in the form of met-enkephalin whereas large molecular weight precursors predominate in adrenal extracts. The proportion of large molecular weight precursor NPY compared to mature NPY in the rat adrenal could be altered by pharmacological manipulation. Thus, acute stress following reserpine treatment resulted in rapid depletion of any measurable large molecular weight precursor, thus suggesting that in some tissues the rate of conversion of the precursor to free peptides may be the limiting factor.

SUMMARY

The original description of NPY following its isolation[3] commented on its homology to the pancreatic polypeptide family of peptides. This homology is extended to the mRNA sequences and the genomic structure, suggesting that this family has arisen as a result of gene duplication. However, each member demonstrates remarkable specificity in its expression within tissue types. The expression of the NPY gene is thus controlled by, as yet undefined, factors regulating to neural cells. The level of expression within cells is dependent on nerve growth factor. However, other factors, such as the rate of processing of the precursor, may be involved in regulation of the stored concentrations of the peptide product.

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Regulation of the Human Neuropeptide Y Gene

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Eukaryotic gene promoters contain *cis*-acting elements that regulate transcription directed by RNA polymerase II. These elements usually interact with sequence-specific DNA-binding proteins which either repress or activate transcription. Typically a number of DNA-binding proteins are capable of interacting with the 5′-flanking sequences of a specific gene. The availability and activity of these proteins determine the extent to which they participate in transcriptional regulation.

The pattern of specific gene expression which generates the complexity demonstrated by the nervous system is not well understood. Because the neuropeptide Y (NPY) gene is expressed widely throughout the central and peripheral nervous systems, it provides a useful model for studying neuronal gene expression.[1-4] NPY is coordinately expressed with a variety of neurotransmitters and neuropeptides including norepinephrine,[5] vasoactive intestinal peptide,[6] acetylcholine,[6] growth hormone releasing hormone,[7] FMRFamide,[8] somatostatin,[9] and cholecystokinin.[10] How individual neurons selectively express various combinations of these neuropeptides is unknown. In order to elucidate the mechanisms underlying neuronal gene expression, we initiated a study on the NPY gene.

A variety of agents such as forskolin, TPA, and NGF influence the steady state levels of rat NPY mRNA,[11,12] suggesting that the NPY gene promoter may be regulated by a combination of transcriptional elements. Furthermore, the 5′-flanking DNA of the human gene contains partial consensus sequences for DNA-binding proteins such as SP1, AP1, and CAAT.[13-17] In combination, several transcriptional elements could generate promoter diversity, culminating in the expression pattern of the NPY gene in the nervous system.

Sequence and Structural Analysis of the NPY Promoter

The 5′-flanking DNA of the NPY gene is shown in FIGURE 1. The sequence displays an unusually distinct partitioning of AT versus GC nucleotides. The distal region (−1078 to −796) contains 67% AT nucleotides while the sequence closer to the start of transcription (−246 to +1) contains only 25% AT nucleotides. A computer program designed to calculate regions of DNA secondary structure predicts that the AT-rich region centered around −780 could potentially form bent DNA.[18,19] Experiments described in a later section demonstrate that deletion of this AT-rich region increases promoter activity. The ability of the AT-rich sequences to participate in negative regulation of the NPY gene is currently under investigation.

Further analysis of the 5′-flanking sequences identified consensus sequences for sev-

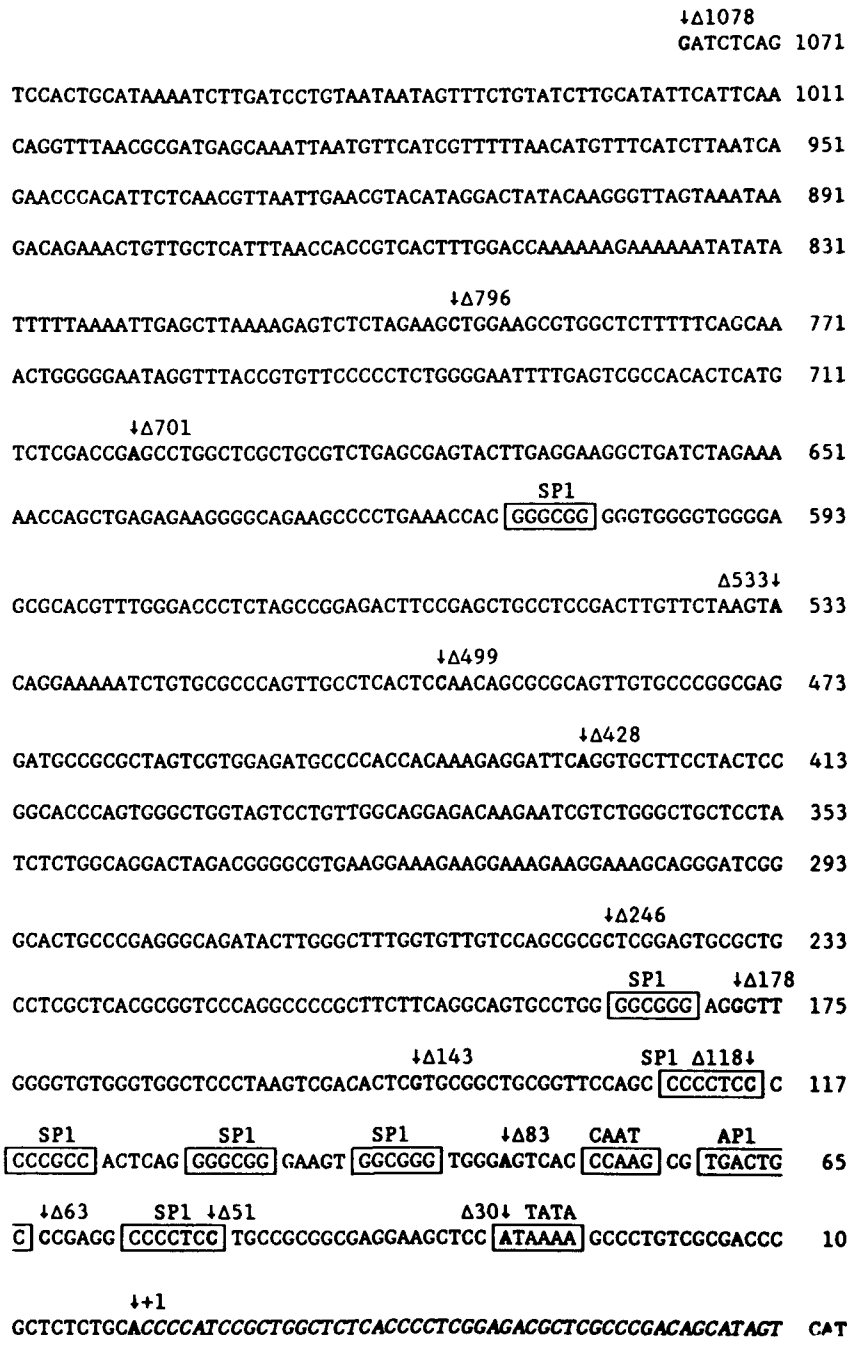
↓Δ1078
GATCTCAG 1071
TCCACTGCATAAAATCTTGATCCTGTAATAATAGTTTCTGTATCTTGCATATTCATTCAA 1011
CAGGTTTAACGCGATGAGCAAATTAATGTTCATCGTTTTTAACATGTTTCATCTTAATCA 951
GAACCCACATTCTCAACGTTAATTGAACGTACATAGGACTATACAAGGGTTAGTAAATAA 891
GACAGAAACTGTTGCTCATTTAACCACCGTCACTTTGGACCAAAAAAGAAAAAATATATA 831
↓Δ796
TTTTTAAAATTGAGCTTAAAAGAGTCTCTAGAAGCTGGAAGCGTGGCTCTTTTTCAGCAA 771
ACTGGGGGAATAGGTTTACCGTGTTCCCCCTCTGGGGAATTTTGAGTCGCCACACTCATG 711
↓Δ701
TCTCGACCGAGCCTGGCTCGCTGCGTCTGAGCGAGTACTTGAGGAAGGCTGATCTAGAAA 651
SP1
AACCAGCTGAGAGAAGGGGCAGAAGCCCCTGAAACCAC GGGCGG GGGTGGGGTGGGGA 593
Δ533↓
GCGCACGTTTGGGACCCTCTAGCCGGAGACTTCCGAGCTGCCTCCGACTTGTTCTAAGTA 533
↓Δ499
CAGGAAAAATCTGTGCGCCCAGTTGCCTCACTCCAACAGCGCGCAGTTGTGCCCGGCGAG 473
↓Δ428
GATGCCGCGCTAGTCGTGGAGATGCCCCACCACAAAGAGGATTCAGGTGCTTCCTACTCC 413
GGCACCCAGTGGGCTGGTAGTCCTGTTGGCAGGAGACAAGAATCGTCTGGGCTGCTCCTA 353
TCTCTGGCAGGACTAGACGGGGCGTGAAGGAAAGAAGGAAAGAAGGAAAGCAGGGATCGG 293
↓Δ246
GCACTGCCCGAGGGCAGATACTTGGGCTTTGGTGTTGTCCAGCGCGCTCGGAGTGCGCTG 233
SP1 ↓Δ178
CCTCGCTCACGCGGTCCCAGGCCCCGCTTCTTCAGGCAGTGCCTGG GGCGGG AGGGTT 175
↓Δ143 SP1 Δ118↓
GGGGTGTGGGTGGCTCCCTAAGTCGACACTCGTGCGGCTGCGGTTCCAGC CCCCTCC C 117
SP1 SP1 SP1 ↓Δ83 CAAT AP1
CCCGCC ACTCAG GGGCGG GAAGT GGCGGG TGGGAGTCAC CCAAG CG TGACTG 65
↓Δ63 SP1 ↓Δ51 Δ30↓ TATA
C CCGAGG CCCCTCC TGCCGCGGCGAGGAAGCTCC ATAAAA GCCCTGTCGCGACCC 10
↓+1
GCTCTCTGCACCCCATCCGCTGGCTCTCACCCCTCGGAGACGCTCGCCCGACAGCATAGT CAT

Fig. 1

eral previously characterized DNA-binding proteins. There are five predicted SP1 GC boxes located at −607, −181, −111, −99, and −88. The region between −86 and −62 is an imperfect palindrome which contains partial CAAT and AP1 consensus sequences. In addition, two CCCCTCC sites are present at −118 and −51. The importance of these CT regions is addressed in the following section.

Deletion Analysis of the 5′-Flanking DNA

A deletion series of the 5′-flanking DNA of the NPY gene was constructed by Exonuclease III digestion of the parent plasmid pCatNPY1078.[20] A schematic diagram depicting the construction of these fusion plasmids is shown in FIGURE 2. These constructs represent varying amounts of 5′-flanking sequences fused to the bacterial reporter gene, chloramphenicol acetyltransferase (CAT). These plasmids were transiently introduced into the human Lan-5 cell line by a modified $CaPO_4$ technique.[21] pRSVLacZ was included as a control to minimize variations between individual transfections and experiments. The Lan-5 cell line affords an ideal system for studying NPY gene expression because it was established from a human neuroblastoma and expresses authentic NPY. The relative CAT conversion for a selected set of fusion constructs is shown in FIGURE 3. These data demonstrate the complexity of NPY gene expression in this system. The increase in expression seen when sequences between −1078 to −796 and −428 to −246 are deleted suggest that these regions may contain negative-acting regulatory elements.

This analysis demonstrated that the sequences necessary for expression of the NPY gene are contained between −246 and −51. A second deletion series spanning this region was constructed and tested (FIG. 4A). The consensus binding sites for several previously characterized DNA-binding proteins are shown in FIGURE 4B. Deletion of the sequence between −63 and −51 abolishes transcription of this gene. Deletion of the CAAT- and AP1-like binding sites at −73 and−64 and the three SP1 consensus binding sites between −118 and −83 do not result in a measurable change of expression. However, deletion of the sequence −143 to −118 which contains another CCCCTCC site results in approximately a 2-fold decrease in CAT activity. This data suggests that the CCCCTCC sequences present in the 5′-flanking DNA are important for NPY gene expression.

Gel Retardation Analysis of the Proximal 5′-Flanking Sequences

The promoter sequence between −75 to −46 was examined for its ability to form specific DNA-protein interactions using the gel retardation assay.[22,23] The nucleotide sequence of the region between −75 and −46 and that of mutant NPY(1) are depicted in FIGURE 5A. A typical gel retardation analysis using this fragment is shown in FIGURE 5B. Two major DNA/protein complexes labelled N1 and N2 are formed in the absence of competitor oligonucleotides (lanes 1 and 20). The oligonucleotides used in the competition reactions contain several defined DNA/protein consensus binding sites. The somatostatin promoter fragment contains a binding site for the cyclic AMP responsive binding protein (CREB).[24] The TRH oligonucleotide contains both a CRE-like sequence (−51)

FIGURE 1. Nucleotide sequence of the human NPY promoter. Nucleotides are numbered from the initiation of transcription and negative values indicate 5′-flanking sequences. Deletion endpoints are numbered and designated by an *arrow* and *bold print*. The TATA consensus sequence is *boxed* as are the consensus sequences for the DNA-binding proteins SP1, AP1, and CAAT.

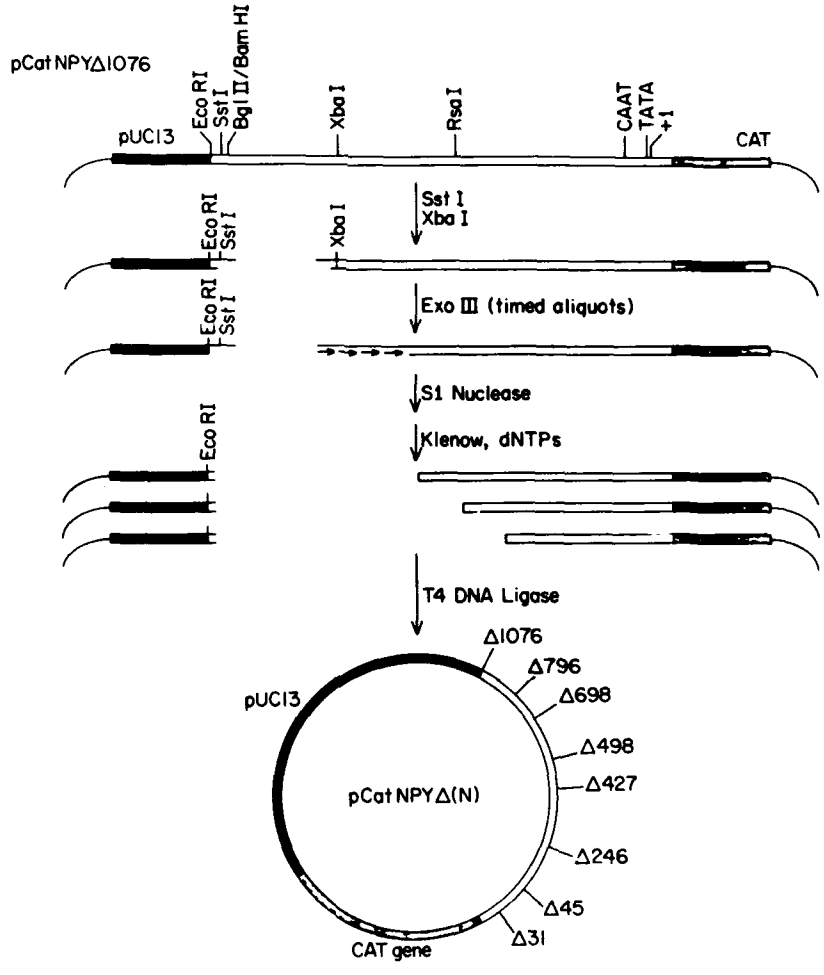

FIGURE 2. Schematic illustration of the construction of the NPY/CAT fusion constructions. The starting plasmid pCatNPY1076 was digested with SstI and XbaI followed by treatment with Exonuclease III for varying times. The ends were blunted by S1 nuclease and repaired with Klenow. Sequence analysis of plasmids obtained from various time points confirmed the deletion endpoints.

and a CT-rich sequence (−62).[25] The human cardiac α- actin and the HSV thymidine kinase promoters contain SP1 consensus binding sites.[26,27] The N2 complex is most efficiently competed when a 500-fold excess of the unlabeled NPY (−75 to −46) or TRH (−70 to −41) oligonucleotide is included in the reaction mixture. The large amount of competitor oligonucleotides needed to reduce the intensity of N2 suggests that this interaction is not specific. However, since all fragments are not capable of competition, a potential binding site at the 5′ end of this oligonucleotide can not be ruled out. The N1 complex is efficiently competed by the cardiac α-actin and the HSV thymidine kinase probes. Furthermore, the mutated NPY oligonucleotide (1) was no longer able to compete

for N1 binding suggesting a DNA/protein interaction with the 3′ end of this oligonucleotide.

Since the addition of nucleotides between −63 and −51 were shown to be important for transcription *in vivo* and the gel retardation analysis indicated that a DNA/protein interaction occurred at this site, another oligonucleotide which corresponded to this region was synthesized and tested. The sequence of this oligonucleotide and that of NPY mutant (2) is shown in FIGURE 6A. The two major complexes formed in this case are designated N1 and N2. As seen in the previous example, N2 is less efficiently competed than the N1 complex and potentially represents a nonspecific interaction. The band between N1 and N2 is not labeled as its intensity varied between extract preparations. The fragments which efficiently compete with the N1 complex contain SP1 binding sites. The SV40 fragment which competes the most efficiently contains two high-affinity SP1 binding sites. We therefore conclude that a SP1-like molecule is capable of binding to the nucleotides CCCCTCC present at −51 of the NPY promoter and that this interaction is important for transcription of this gene.

In Vitro *Transcription Directed from the NPY Promoter*

In order to develop a functional assay for the NPY promoter elements, high efficiency nuclear transcription extracts were prepared from Lan-5 cell nuclei.[28] The Adenovirus major late promoter (Ad5 MLP) sequences −270 to +32 were included in all transcription reactions to serve as an internal control. The NPY/CAT fusion plasmids were identical to those used in the *in vivo* transfection studies. The amount of RNA synthesized from each template was measured by primer extension analysis utilizing a primer com-

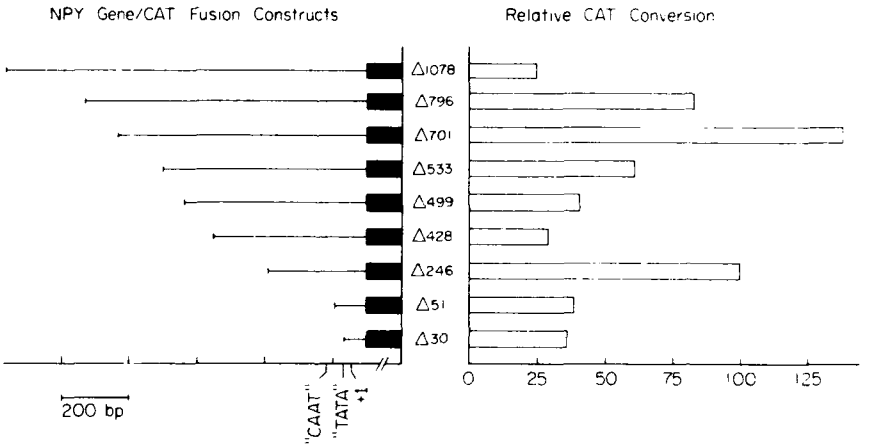

FIGURE 3. Transient expression of NPY/CAT fusion constructions. *Left:* Schematic diagram of the 5′-flanking sequences for the NPY/CAT deletion constructions. The *solid box* represents 51 nucleotides of the first nontranslated exon of the NPY gene, the coding sequences for the CAT structural gene, the SV40 small T antigen intervening sequence, and the SV40 polyadenylation signal. The *thin line* represents NPY promoter sequences. *Right:* Relative CAT conversion obtained from transient transfection of Lan-5 cells with the corresponding NPY fusion construct depicted to the left and the control plasmid pRSVLacZ. These values are a ratio of CAT/A_{420} nm. The activities are presented relative to the −246 deletion. Data represent an average of 2–5 transfections for each construct.

plementary to the CAT gene (FIG. 7A). Correct initiation of transcription results in bands of 131 and 110 nucleotides in length for the NPY and the MLP transcripts, respectively (FIG. 7B). Correct initiation of RNA synthesis from the various promoter deletions used in this system correlate with function in the transient transfection analyses. Two notable

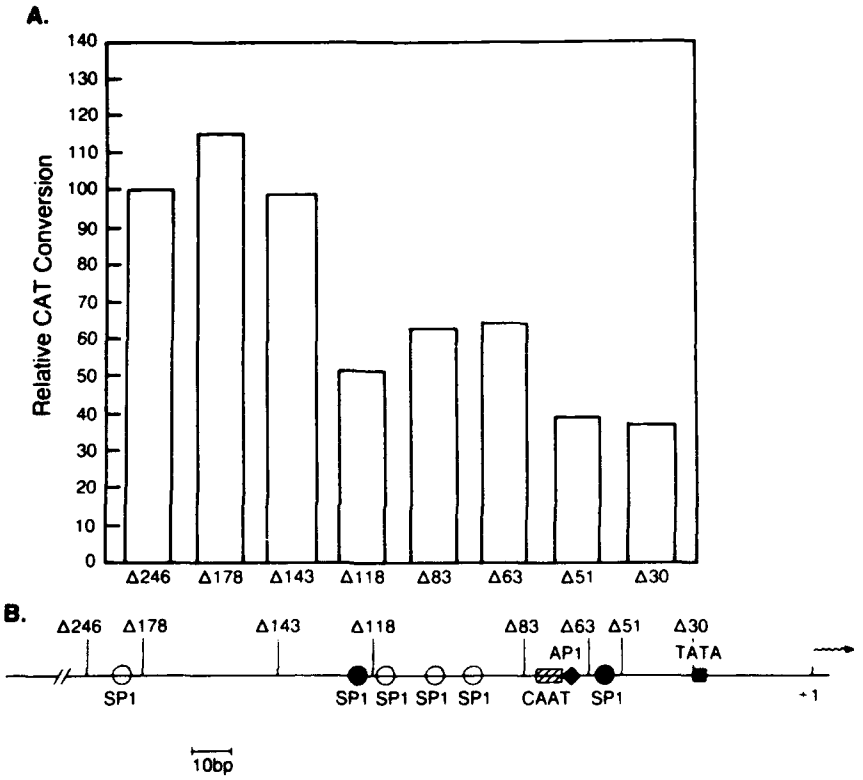

FIGURE 4. Transient expression of proximal NPY/CAT fusion constructions. **(A)** The *open bars* represent the relative CAT conversion obtained from transient transfections of Lan-5 cells with the deletions ranging from −246 to −30 and the plasmid pRSVLacZ. The values are a ratio of CAT/A_{420} nm relative to the −246 deletion. **(B)** Schematic diagram of *cis*-acting promoter elements between −246 and −30. The positions of the deletions are denoted by *vertical lines*. The TATA box is represented as a *blackened box*. The CCCCTCC sequences are depicted by *blackened circles* while the GGGCGG SP1 consensus sequences are represented by *open circles*. The putative AP1 and CAAT consensus sequences are represented by a *blackened diamond* and a *hatched box*, respectively.

differences are the −30 and −51 constructs. Both of these plasmids were capable of generating significant CAT activity *in vivo*, but were unable to direct the correct initiation of NPY mRNA *in vitro*. In addition, the −1078 construct, whose CAT activity was consistently less than that of the −51 and −30 constructs in the *in vivo* experiments, is capable of accurate transcription of the NPY promoter *in vitro*. These experiments dem-

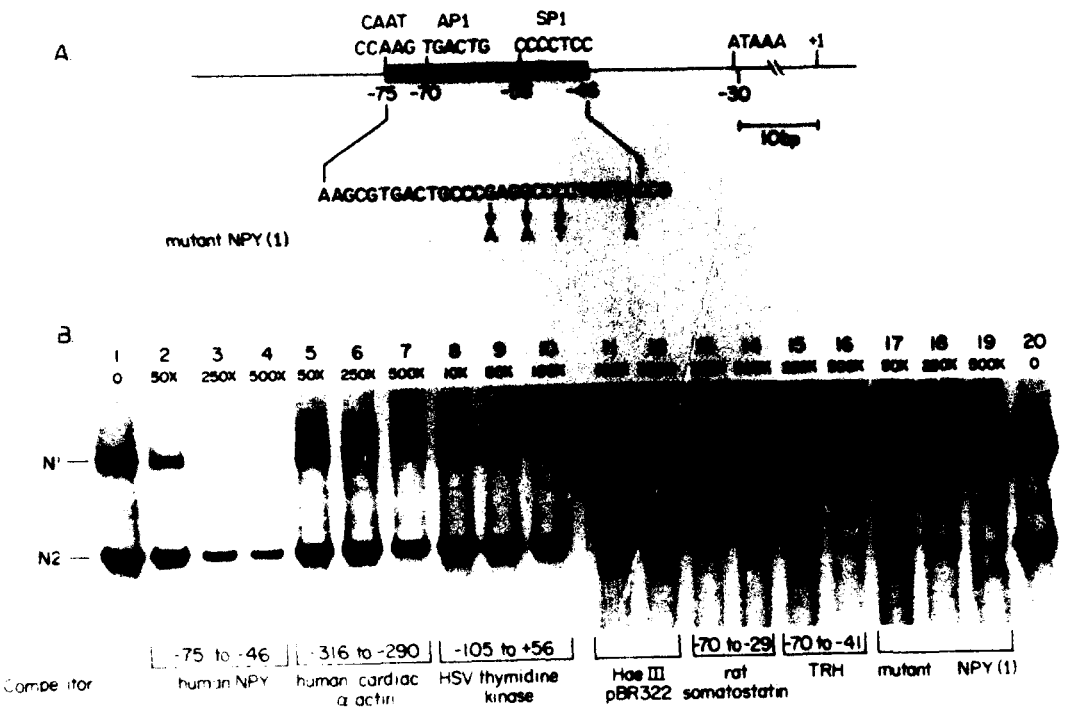

FIGURE 5. Gel retardation analysis of NPY promoter. **(A)** The sequence of the NPY promoter between −75 and −46. The CAAT, AP1, and SP1 binding sites are labeled and the nucleotide changes in the mutant NPY (1) oligonucleotide are noted by *arrows*. B. Lanes 1 and 20 represent the gel-retardation pattern obtained with Lan-5 nuclear extract and the oligonucleotide (−75 to −46) when no competitor DNA is present. The competitor DNAs present in lanes 2–19 are listed *below* the lanes in which they appear. The fold excess of each competitor is noted *above* the lane number. The two major complexes formed are designated N1 and N2.

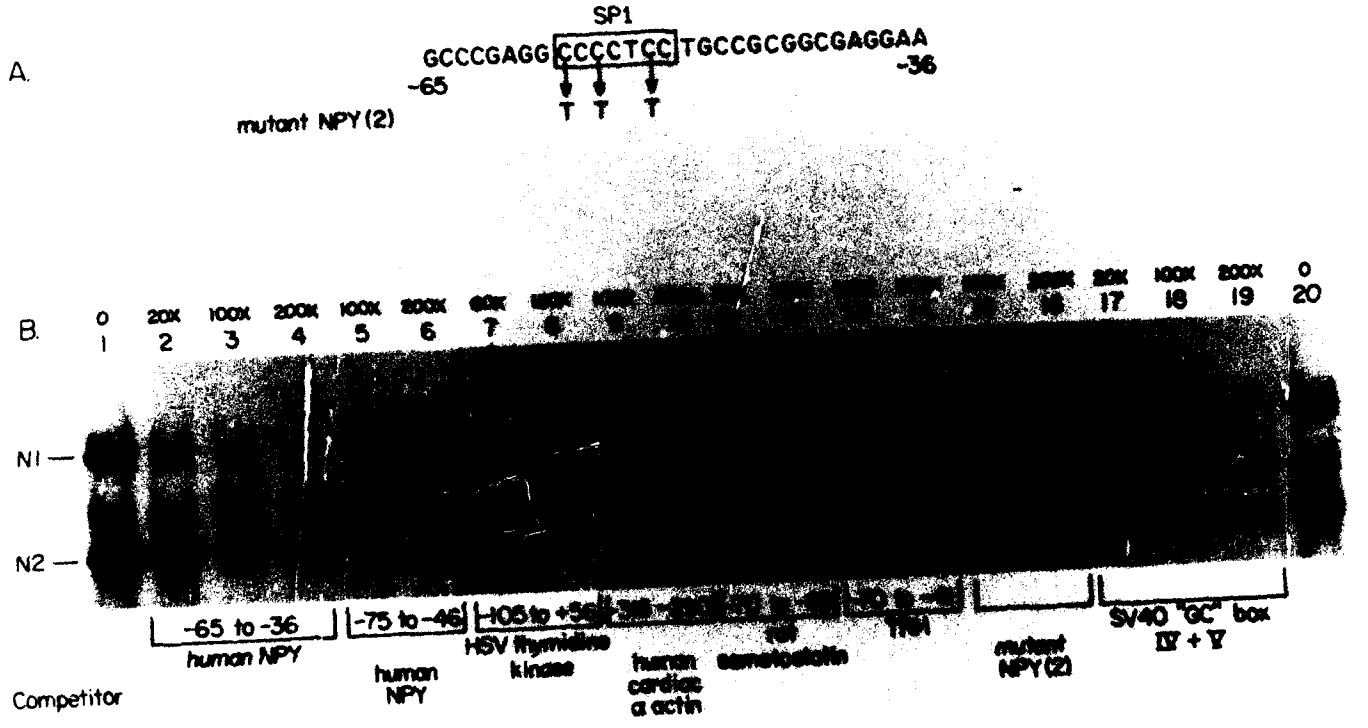

FIGURE 6. Gel retardation analysis of NPY promoter. **(A)** The sequence of the NPY promoter between −65 and −36. The SP1 binding site is *boxed* and the C to T nucleotide changes in the mutant NPY (2) oligonucleotide are noted by *arrows*. **(B)** Lanes 1 and 20 represent the gel-retardation pattern obtained with Lan-5 nuclear extract and the oligonucleotide (−65 to −36) when no competitor DNA is present. The competitor DNAs present in lanes 2–19 are listed *below* the lanes in which they appear. The fold excess of each competitor is noted *above* the lane number. The two major complexes formed are designated N1 and N2.

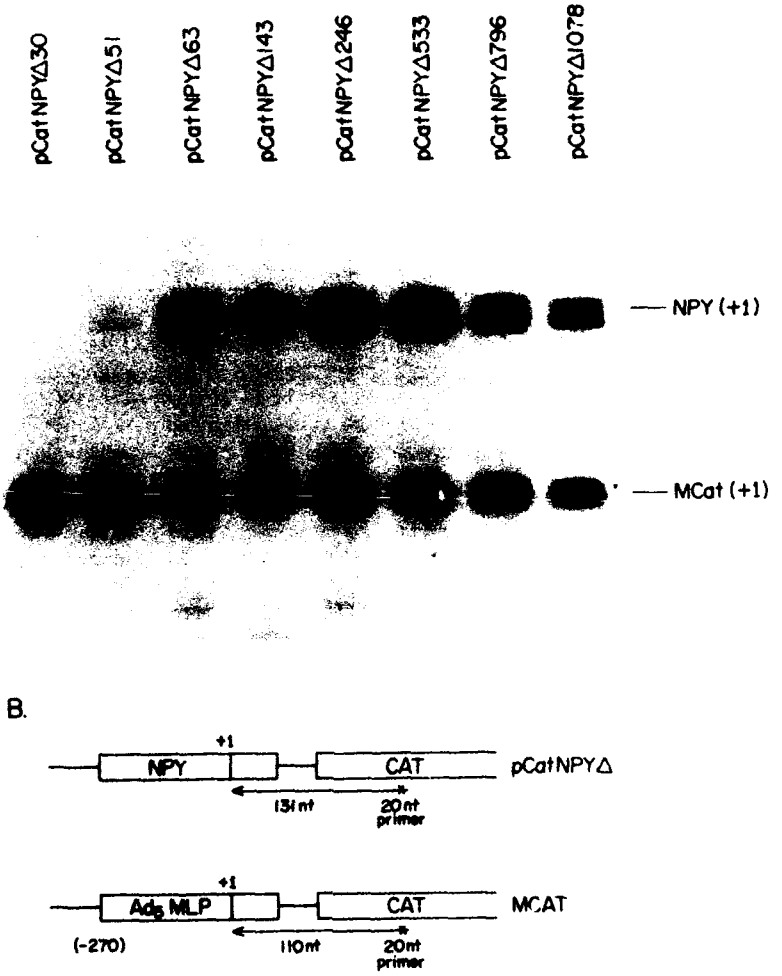

FIGURE 7. *In vitro* transcription analyses. **(A)** *In vitro* transcription products directed from the NPY and MCAT promoters. Each reaction contains 3 mg/ml Lan-5 nuclear extract, 0.75 μg of the designated NPY-CAT fusion constructs and 0.25 μg of MCAT. The positions of the correctly initiated primer extension products for the NPY and MCAT constructs are noted to the *right*. **(B)** Schematic diagram of the templates utilized in the *in vitro* transcription analyses. Templates pCat-NPY- and MCAT contain various amounts of the 5′-flanking sequences of the NPY promoter and Ad5 MLP, respectively, fused to the bacterial CAT gene. The primer is complementary to the sequences +15 to +35 of the CAT transcript. The *arrow* depicts the predicted primer-extension products arising from the *in vitro*-synthesized RNA. nt, nucleotide.

onstrate the importance of correlating CAT activities with correctly initiated transcription from the NPY promoter and indicate that vector sequences placed proximal to promoter sequences can generate spurious transcripts resulting in exaggerated CAT activities *in vivo*.

In Vitro *Extract Depletion Experiment*

The sequence between −63 and −51 is important for NPY gene expression based on both *in vivo* transfections and *in vitro* transcriptions. We demonstrated that a SP1-like molecule is capable of binding to this region. To test whether this molecule is necessary to direct expression of the NPY gene, *in vitro* extract depletion experiments were performed. The construct pCatNPY246 is used as the template for the reactions because it is consistently expressed at a high level in both the *in vitro* and *in vivo* experimental systems. In the depletion reactions, 300 ng of competitor oligonucleotide was added prior to the addition of Lan-5 nuclear extract. Both the NPY promoter sequence −65 to −36 and the SV40 promoter GC boxes IV and V significantly decreased transcription of the NPY promoter (FIG. 8). The NPY mutant oligonucleotide (2) which no longer competes for protein binding in the gel retardation experiments was incapable of decreasing transcription. This competition was specific as addition of excess SP1 competitor oligonucleotides did not inhibit transcription directed by the Ad5 MLP. The autoradiograph was scanned with a densitometer and the values for NPY/CAT normalized to those for MCAT. The SV40 fragment decreased transcription to 15% of the control while the NPY promoter fragment decreased transcription to 20% of the control. These results demonstrate that a SP1-like molecule specifically acts as a transactivator of the NPY gene.

A number of studies have demonstrated that interactions involving the binding of multiple proteins to neighboring promoter sequences are required for promoter activation.[26,27,29] The sequences responsible for NPY expression contain CT-rich binding sites which interact with SP1-like proteins. These sites are not representative of the typical SP1 binding sites (TABLE 1). Many other promoters contain SP1 binding sites. For example, SP1 is important for expression of the TK and the immediate early genes of herpes simplex virus as well as the LTR of the HIV retrovirus.[27,30,31] SP1 also activates the promoters of the mouse DHFR,[32] mouse hypoxanthine phosphoribosyl transferase,[33] mouse adenine phosphoribosyl-transferase,[34] rat type II procollagen,[35] human metallothionein IIA,[29] hamster hydroxymethyl glutaryl CoA reductase,[36] human c-Ha-ras 1,[37] human adenosine deaminase,[38] human urokinase-plasminogen activator,[39] and human cardiac α-actin genes.[26] The CCCCTCC sites in the NPY promoter most closely resemble the SP1-binding sites in the cardiac α-actin promoter. The role SP1 plays in the transcriptional activation of such a diverse group of genes has yet to be elucidated.

Tissue specificity may be controlled by subtle differences in transcription factors. SP1

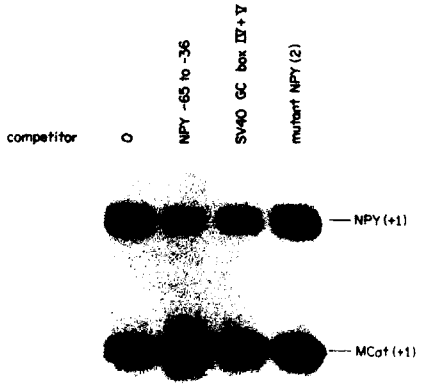

FIGURE 8. *In vitro* transcription competition analyses. The ability of Lan-5 nuclear extract to transcribe the templates pCatNPY246 and MCAT was assayed in the presence and absence of 0.3 μg of competitor DNA fragments. The competitor fragments are listed *above* the lanes in which they appear. The positions of correctly initiated NPY and MCAT transcripts are noted to the *right*.

TABLE 1.[a] Potential SP1 Binding Sites in the hNPY Promoter

SP1 consensus		G G G G C G G G G C T A A T
NPY promoter GC sequences	(−95)	G *T* G G C G G G *T G* (−86)
	(−105)	G G G G C G G G A *A* (−96)
	(−117)	C C C C G C C *A* C *T* (−108)
	(−188)	G G G G C G G G A *G* (−179)
	(−613)	*C* G G G C G G G G *G* (−604)
NPY promoter CT sequences	(−57)	C C C C T C C T G C (−48)
	(−124)	C C C C T C C C C C (−115)

[a]*In* the case of the GC-rich sequences, nucleotides which vary from the consensus are *italicized*.

isolated from HeLa cells fits into this type of model as it belongs to a family of proteins which have been shown to be posttranslationally modified by O-glycosylation. This modification was shown to be important for the transcriptional activation elicited by SP1.[40] However, it does not account for all the forms of HeLa cell SP1 proteins. Other modifications or related molecules must contribute to family diversity. In addition, neuronal SP1 family members have yet to be characterized. Subtle differences in these proteins may contribute to their ability to activate the transcription of specific genes.

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PART II. ROLE OF NEUROPEPTIDE Y IN THE REGULATION OF THE CIRCULATORY SYSTEM

Neuropeptide Y and Central Cardiovascular Regulation

Focus on Its Role as a Cotransmitter in Cardiovascular Adrenergic Neurons[a]

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INTRODUCTION

Especially during the eighties the concept has been established that communication within the central nervous system involves not only the classical synaptic transmission (wiring transmission, WT) but also a transmission which involves the diffusion of released transmitters in the extracellular fluid pathways to reach distant high affinity receptors (volume transmission, VT).[1,2] Several observations have led to the development of this concept such as the demonstration of lack of synaptic specializations in many monoamine nerve terminals of the brain,[3] nonsynaptic release,[4] demonstrations of extraneuronal catecholamines (CA) and 5-hydroxytryptamine (5-HT), and their rapid diffusion within the extracellular fluid.[5–8] The demonstration of marked receptor-transmitter mismatches in the CNS has also been interpreted to reflect the existence of VT in the brain.[1,9,10] The neuropeptides appear to be highly suitable to participate in VT, since they are not taken up again by the nerve terminals once released and since active fragments may be formed as the actions of peptidases in the extracellular fluid. The active fragments can, via their respective receptors, exert positive or negative feedback actions on the original biological responses induced by the parent peptide as well as produce comple-

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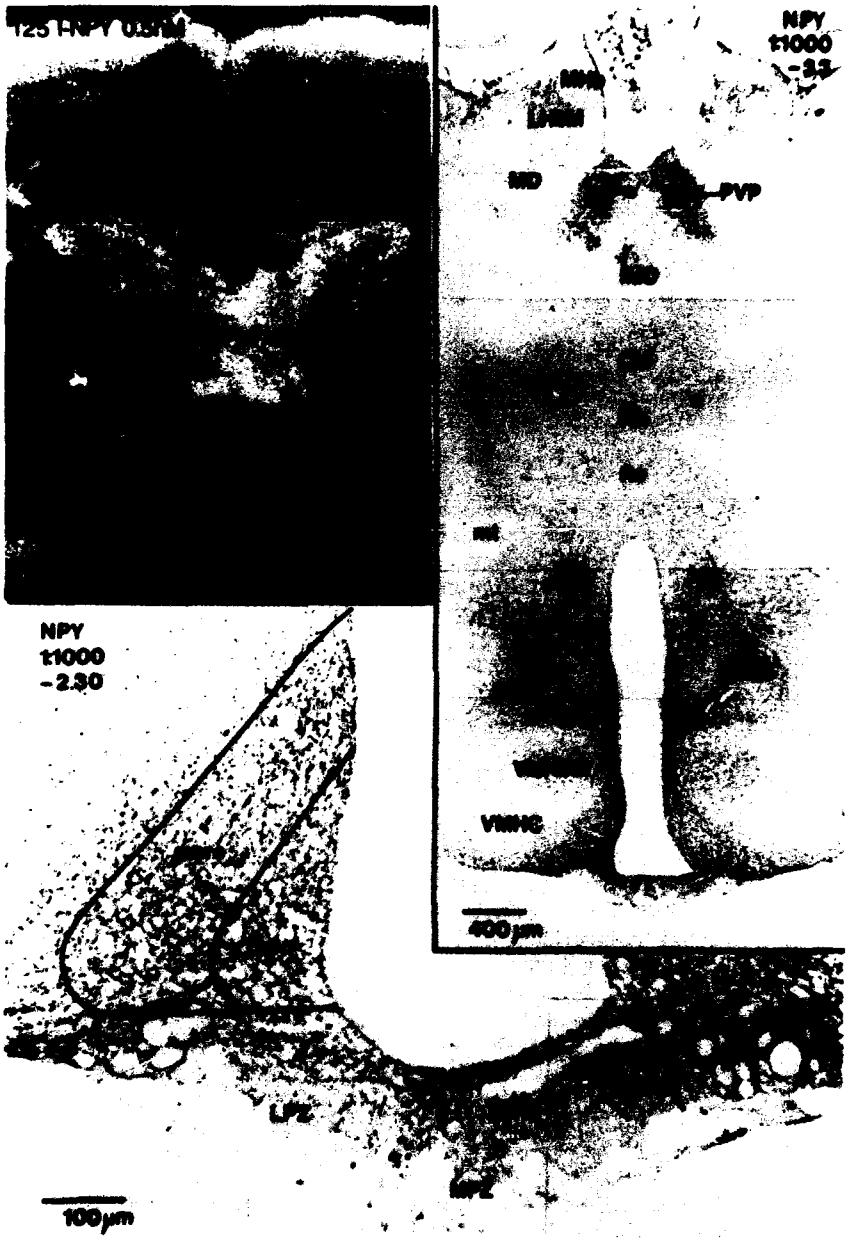
NPY
1:1000
-2.30
400 µm
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mentary responses. Thus, in VT, chemical networks may be formed, at least as far as neuropeptides are involved, leading to the development of syndromic responses.[11–15]

Central neuropeptide Y (NPY) neurons appear to an important extent to communicate with other cells of the brain via VT in view of the topological and functional transmitter receptor mismatches (FIG. 1) demonstrated in this system.[12,16–19] Furthermore, we have been able to obtain evidence that the high affinity NPY receptors in mismatch areas represent biologically active NPY receptors.[19,20] In the present article we will discuss communication in the cardiovascular NPY/adrenaline costoring neurons,[21,22] especially in relation to WT and VT. We will also present evidence that the NPY fragment porcine NPY 13–36 (pNPY 13–36) upon central administration can produce vasopressor actions mediated by activation of NPY receptors of the Y2 type.[23] It is postulated that such fragments can be formed *in vivo* to counteract the vasodepressor response of the pNPY 1–36,[24] which is mediated through activation of Y1 receptors.[14,25,26] In addition, we will discuss NPY/adrenaline cotransmission in central cardiovascular regulation where the two transmission lines may interact via NPY/α2 receptor interactions in the plasma membrane, both at the pre- and postsynaptic level. These receptor-receptor interactions may help to facilitate a switching between VT and WT. We will also summarize recent evidence that angiotensin peptides may serve as cotransmitters in NPY/adrenaline and in NPY/noradrenaline (NA) systems of cardiovascular regions.[27] Finally, the evidence will be discussed that adrenalectomy leads to differential actions on adrenaline and NPY cotransmission in the medulla oblongata.[28]

Anatomy of NPY/Adrenaline Costoring Cardiovascular Neurons

The central adrenaline neurons were discovered by Hökfelt, Fuxe, Johansson and Goldstein in 1973.[29] NPY-like immunoreactivity (IR) was originally demonstrated in these neurons by Hökfelt and colleagues in 1983.[30,31] The NPY/adrenaline costoring cell bodies are mainly located in the rostral medulla, especially in the ventrolateral area (group C1),[29] and these adrenergic neurons project to cardiovascular areas of the brain, including the nucleus tractus solitarius (nTS) and the sympathetic lateral column of the spinal cord.[32–34] Subsequently, a small local NPY/adrenaline costoring system was discovered within the cardiovascular region of the nTS (dorsal strip).[32,33,35] By means of newly developed microdensitometrical and morphometrical methods[36–38] it has become possible to quantitatively evaluate the degree of coexistence of phenylethanolamine-

FIGURE 1. Autoradiogram of ^{125}I-NPY (0.5 nM) labelling with corresponding NPY IR in the area of thalamus and hypothalamus at Bregma level -2.3 mm. A strong NPY binding is present in PVP, MD, IMD, CM and Re, while a moderate NPY binding is present in MHb, LHbM, VMHDM, VMHC and MPZ. A dense plexa of NPY IR is seen in PVP, DA, DMC and DMD, whereas moderate density of plexa is present in LHbM, VMHDM, marc, Parc, LPZ, MPZ and very few scattered if hardly any NPY IR terminals are present in MHb, MD, CM, Rh, Re and VMHC. Matches correspond predominantly to PVP, IMD and MPZ where both high density of binding and IR terminals are seen. In the area of MHb, LHbM, VMHDM matches are also present with low to moderate density of both terminals and binding. Mismatches with high-density binding and very few if any NPY IR terminals are seen in MD, CM, Rh, Re and VMHC. Abbreviations: CM, central medial thalamic nucleus; DA, dorsal hypothalamic area; DMC, dorsal hypothalamic nucleus, compact part; DMD, dorsal hypothalamic nucleus, diffuse part; IMD, intermediodorsal thalamic area; LHbM, lateral habenular nucleus, medial part; LPZ, lateral palisade zone; MD, mediodorsal thalamic nucleus; MHb, medial hebenular nucleus; PVP, paraventricular thalamic nucleus, posterior part; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; VMHC, ventromedial hypothalamic nucleus, central part; VMHDM, ventromedial hypothalamic nucleus, dorsomedial part.

N-methyltransferase (PNMT) and NPY in adrenaline cell bodies and nerve terminals.[37] The studies reveal a high degree of coexistence of NPY and PNMT IR within nerve terminals in cardiovascular regions such as the nTS and dorsal motor nucleus of the vagus.[12] In a morphometrical analysis the NPY/adrenaline costoring neurons *inter alia* show a preferential disappearance of NPY IR during aging in certain cardiovascular adrenaline neurons, especially those of the C1 area.[39] Thus, in aging the neurons may not simply degenerate but may show a progressive impairment in their functional properties; in the case of group C1 a reduction of the cotransmitter NPY. Taken together, these results are compatible with the existence of NPY/adrenaline cotransmission in cardiovascular regions of the brain such as the dorsal medulla and the sympathetic lateral column of the spinal cord.

On the Existence of NPY Receptors in Central Cardiovascular Areas

Large numbers of high-affinity ^{125}I-NPY 1–36 binding sites have been mapped out within the CNS of the rat brain.[12,25,40,41] We have recently demonstrated marked transmitter-receptor mismatches within the various central NPY neuronal systems, including

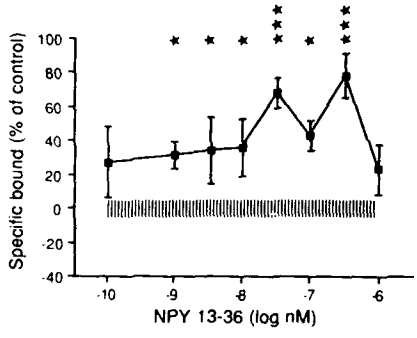

FIGURE 2. Effects of the pNPY 13–36 fragment on ^{125}I-NPY (0.4 nM) binding in the nucleus centro medialis of the thalamus using quantitative receptor autoradiography (see legend in FIG. 12). Data represent % of control value ± SEM. Fisher's PLSD-test: $*p < 0.05$, $***p < 0.001$.

the nTS.[16,42] Within the dorsomedial medulla the highest density of high-affinity ^{125}I-NPY 1–36 binding sites is found within the dorsal strip region, while the highest density of NPY IR nerve terminals is demonstrated in the ventral parts of nTS and within the dorsal motor nucleus of the vagus. The results amplify a role of NPY receptors in cardiovascular regulation, since the baroreceptor afferents terminate in the dorsal strip region. Thus, the NPY receptors can modulate transmission at the primary relay station for the baroreceptor afferents.

Recently, two types of NPY receptors have been demonstrated within the CNS, that is NPY receptors of the Y1 type, which can only recognize the full parent peptide, pNPY 1–36, and NPY receptors of the Y2 type, which also can recognize C-terminal fragments of pNPY, especially pNPY 13–36.[23,43,44] It should be pointed out, however that in receptor autoradiograms ^{125}I-NPY 1–36 predominantly labels the Y1 receptors, since the C-terminal pNPY fragment 13–36 only can displace ^{125}I-pNPY 1–36 from its binding sites in very high concentrations (over 1 μM) (FIG. 2) (unpublished data). Therefore, it will be of substantial interest to map out the ^{125}I-NPY 13–36 binding sites in the CNS. Taken together, the available data indicate that the high-affinity NPY receptors of the Y1 type to a large extent may be reached by pNPY 1–36 diffusing in the extracellular fluid and, thus participate in VT.

Neuropeptide Y/α2 Receptor-Receptor Interactions

In both biochemical binding experiments and in receptor autoradiography it has been demonstrated that pNPY, in the nanomolar range, produces a dose-related increase of the K_D value of α2 agonist binding sites within the dorsal medulla using the radioligand ^{3}H-paraminoclonidine.[45–47] Thus, NPY receptors appear to be capable to reduce the affinity of α2 adrenoceptors via intramembrane interactions. This switch is agonist specific, since it was not observed when the α2 adrenoceptor antagonist ^{3}H-idazoxan was used.[46] Furthermore, the GTP-induced shift in the IC_{50} value of clonidine for displacement of ^{3}H-idazoxan from its binding sites was not modulated by pNPY 1–36 (10 nM).[41] These results were compatible with the involvement of the G-proteins in these intramembrane receptor-receptor interactions. In line with this idea was also the demonstration that both NPY and α2 receptors are coupled in an inhibitory way to the adenylate cyclase via a G_i protein.[41] Evidence for this view was recently obtained by the demonstration that pertussin-toxin treatment counteracts intramembrane interactions between NPY receptors and α2 adrenoceptors.[47] Thus, pertussin-toxin treatment counteracted this interaction within the plasma membrane without reducing the binding of NPY to its own receptor.

The interaction between NPY receptors, probably of the Y1 type in the dorsal medulla, and α2 adrenoceptors appears to be bidirectional, since both *in vitro* and *in vivo* clonidine, an α2 agonist, can reduce the binding of ^{125}I-NPY 1–36 within the nTS at various rostrocaudal levels.[46] These results indicate the existence of antagonistic intramembrane receptor-receptor interactions between the NPY receptors of the Y1 type and of the α2 receptors. Thus, it seems possible that in NPY/adrenaline cotransmission in the nTS the two transmission lines can modulate the activity of one another via intramembrane receptor-receptor interactions, which appear to take place both at the presynaptic and the postsynaptic level.[48] However, 1 nM of pNPY has been found to significantly enhance the ability of clonidine to inhibit the potassium-induced release of ^{3}H-noradrenaline (^{3}H-NA) in synaptosomal preparations from the dorsal medulla. Thus, in this case the receptor-receptor interactions appears to lead to an enhanced sensitivity of the α2 autoreceptor. Taken together, these findings are compatible with the idea that the NPY transmission line, can downregulate adrenaline transmission via pre- and postsynaptic modulation of the α2 adrenoceptor. It should also be mentioned that this interaction is no longer present in the spontaneously hypertensive rat (SHR), neither at the pre- nor at the postsynaptic level.[48,49] In the same way, the adrenaline transmission line may downregulate the NPY transmission line in NPY/adrenaline cotransmission.

Central Cardiovascular Actions of NPY and Adrenaline

It was early demonstrated that adrenaline and pNPY when given intraventricularly (i.v.t.) or intracisternally (i.c.) in the awake or the anaesthetized rat can produce a lowering of mean arterial blood pressure (MAP), of heart rate (HR) and of respiration rate.[21,24,50,51] The major difference between adrenaline and pNPY is the long duration of action of NPY versus adrenaline as shown in FIG. 3.[14,52] When pNPY and adrenaline are given together in the cisterna magna they antagonize the cardiovascular actions of one another.[53] These results are in line with the biochemical and receptor autoradiographical studies indicating that in NPY/adrenaline cotransmission the two transmission lines can exert an inhibitory influence on each other.

In the SHR the ED_{50} value of pNPY 1–36 is substantially increased with regard to lowering of MAP as compared to the normotensive rat.[54] These results may reflect an altered sensitivity of the NPY receptor of the Y1 type in the cardiovascular regions of the brain. In line with the postulate that there is an altered sensitivity of these types of NPY receptors in SHR is the demonstration that pNPY 1–36 can no longer modulate the presynaptic or the postsynaptic α2 adrenoceptors in SHR.[48,49]

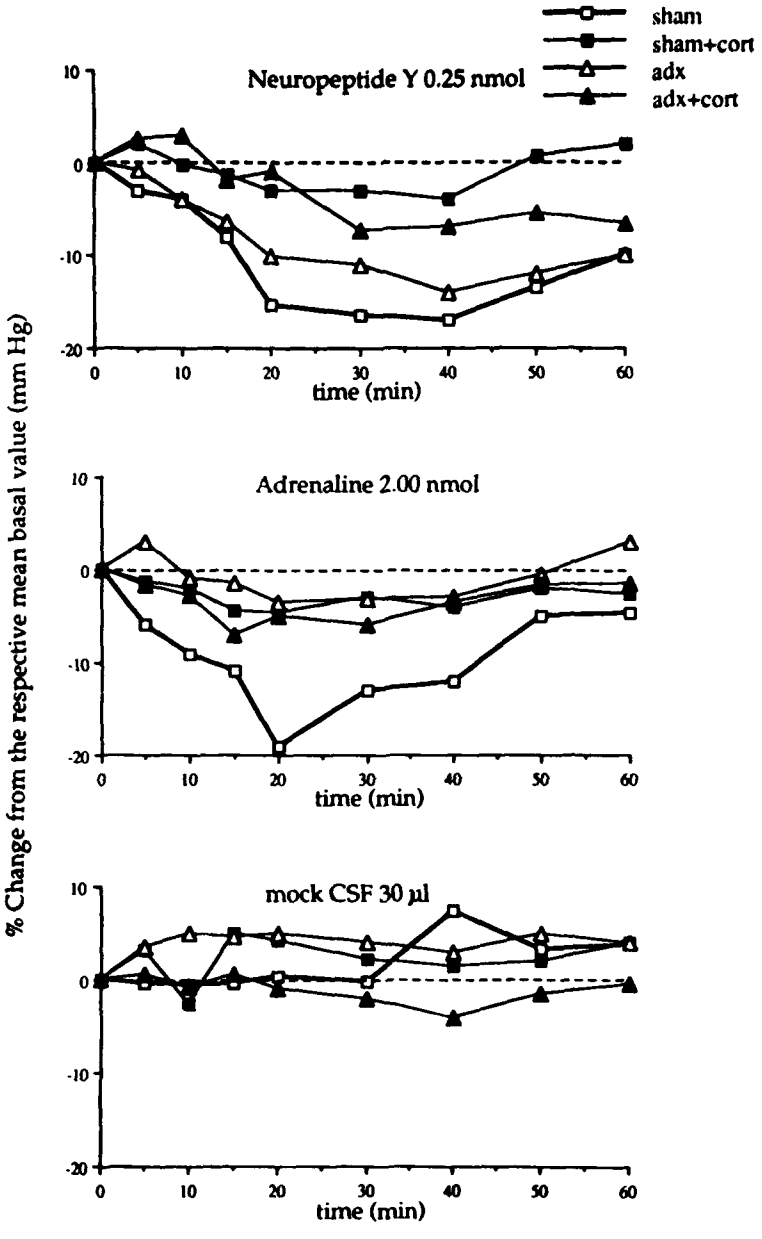

FIGURE 3. Comparisons of the time-course of changes of mean arterial blood pressure after central administration (i.v.t.) of porcine neuropeptide Y 1–36 (pNPY, 0.25 nmol), adrenaline (base) (A, 2.00 nmol) and mock CSF (10 µl/min × 3 min) in awake freely moving male rats with various alterations in pituitary-adrenal activity (sham operated vehicle treated rats = sham + veh; adrenalectomized vehicle treated rat = adx + veh; sham operated corticosterone treated rat = sham + cort and adrenalectomized corticosterone treated rat = adx + cort). The basal values for the mean arterial blood pressure (MAP) (mm Hg): sham + veh (n = 20) = 103 ± 1; sham + cort (n = 22) = 102 ± 1; adx + veh (n = 21) = 100 ± 1; adx + cort (n = 21) = 102 ± 1. Data represent means expressed in percent of the basal value; SEM varied from 1–20%. For statistics in relation with peak effects, see FIGURE 13.

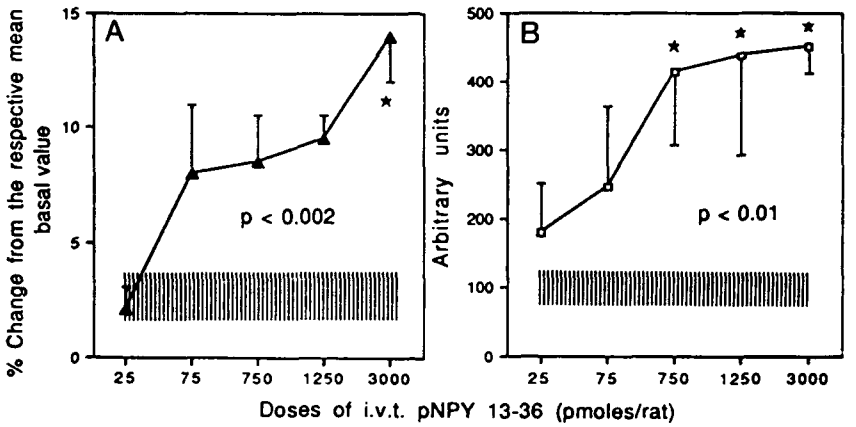

FIGURE 4. Effects on mean arterial blood pressure (MAP; mm Hg) by different doses of the pNPY 13–36 fragment, i.v.t. administered in the awake freely moving male rat. Peak actions (maximal responses) and area values (overall effects) are expressed as means ± SEM. The Joncheree-Terpstra test for ordered alternatives to evaluate dose related changes ($p < 0.01$) and the Treatment *versus* Control nonparametric test (⋆ = $p < 0.05$) were used. *Striped areas* represent the SEM limits for the peak mean increase of MAP by mock CSF alone (10 μl/min × 3 min). Basal values (mm Hg) for each group: mock CSF 30 μl alone = 106 ± 4; pNPY 13–36 25 pmol = 100 ± 5; pNPY 13–36 75 pmol = 99 ± 3; pNPY 13–36 750 pmol = 109 ± 6; pNPY 13–36 1250 pmol = 100 ± 6; and pNPY 13–36 3000 pmol = 100 ± 5.

Cardiovascular Actions of pNPY 13–36 in Normotensive and Spontaneously Hypertensive Rats

Intraventricular injections of 5 doses of the putative Y2 NPY receptor agonist pNPY 13–36 (25–3000 pmoles/rat) in the awake unrestrained freely moving male rat produce a significant dose-related increase in MAP without effects on HR (FIGS. 4 and 5). Thus, in contrast to pNPY 1–36 which activates Y1 receptors, NPY receptor agonists of the Y2 type increase MAP when i.v.t. administered. Thus, NPY receptors of both the Y1 and Y2 type appear to participate in central cardiovascular regulation, where they may oppose the functional consequences of one another. Unlike pNPY 1–36 which possesses a potent bradycardic action, the fragment pNPY 13–36 does not influence HR compared with mock CSF alone. Thus, only NPY receptors of the Y1 type appear to be involved in the regulation of HR.

The potency of pNPY 13–36 to exert a vasopressor action upon i.v.t. injection (FIG. 5) in the awake unrestrained rat was substantially increased in the SHR compared with the Wistar-Kyoto control animal (WKY) (FIG. 6). Thus, a low dose of pNPY 13–36 (25 pmoles/rat) exerted substantial and long-lasting increases of MAP, in SHR but not in the WKY normotensive animal. These results indicate that in the SHR and Y2 receptor sensitivity appears to be increased. Such a disturbance may contribute to the development of the spontaneous hypertension. It may be speculated that the degradation-regulated switch between isoreceptor transmission lines may be disturbed in the SHR leading to an enhancement of the Y2 transmission line.

Possible Y1 and Y2 NPY Receptor Interactions

In receptor autoradiographical experiments it has recently been found that pNPY 13–36 in concentrations of 3–100 nM can increase the binding of ^{125}I-NPY 1–36 (FIG.

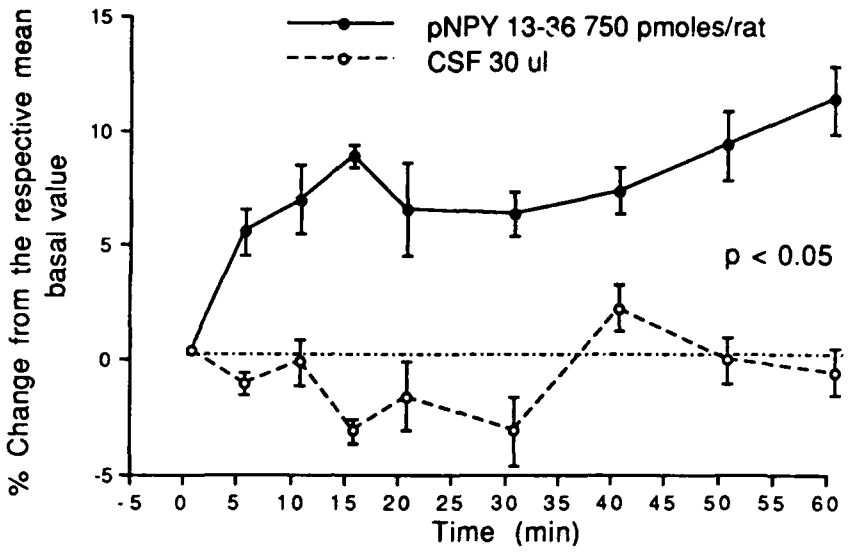

FIGURE 5. Time-curve of the central effects on mean arterial blood pressure (MAP; mm Hg) by i.v.t. pNPY 13–36 (750 pmol). The effects of mock CSF alone (10 μl/min × 3 min) are also shown. Each point is represented as means ± SEM of % change from the MAP basal value for each group of treated rats (for basal values see FIG. 4).

2). Displacement is only noted in a high concentration of 1000 nM. These results can be explained on the basis of the existence of interactions between NPY receptors of the Y1 and Y2 type. One possibility is that the activation of the NPY receptor of the Y2 type may lead to an increase in the affinity of the NPY receptors of the Y1 type. The mechanism underlying such a change in affinity is unknown but may reflect an altered coupling to the G_i-proteins, since it has recently been demonstrated that i.v.t. pertussis-toxin treatment can lead to the development of an increase in the binding of ^{125}I-NPY 1–36 in the dorsomedial medulla.[47] Such an interpretation is also supported by functional studies on interactions between Y1 and Y2 receptors in cardiovascular regulation. Thus, as seen in FIGURE 7, the threshold dose of the pNPY fragment 13–36 can significantly counteract the vasopressor actions of a close to ED_{50} dose of pNPY 1–36. In contrast, the threshold dose of pNPY 1–36 does not modify the pressor action of the pNPY fragment 13–36 using a close to ED_{50} dose[55] (FIG. 8). These results give evidence for the existence of chemical networks in central NPY transmission.[55] Thus, it is mainly the active fragment which has a role in the termination of the biological response. These results indicate that NPY transmission has a high degree of flexibility and is suitable for the long-term regulation of cardiovascular function *inter alia* by its potential ability to form active C-terminal fragments. This strongly supports the view that VT represents an important component in the chemical communication of central NPY neurons.

Neuroendocrine Actions of pNPY 13–36: The Relationship to Effects on Hypothalamic Catecholamine Nerve Terminal Systems

In previous work i.v.t. injections of pNPY 1–36 in doses of 7.5–25 pmoles/rat were demonstrated to produce inhibitory actions on serum levels of corticosterone (CORTICO)

and aldosterone (ALDO) and to increase serum prolactin (PRL) levels in the normal male rat as evaluated within the 15–60 min time interval.[18,56,58] In contrast, in high doses ranging from 250–750 pmol pNPY 1–36 produced inhibitory effects on serum thyreotropine (TSH), PRL and growth hormone (GH) levels, while the serum levels of CORTICO, adrenocorticotropin (ACTH) and ALDO were increased.

It has only been possible to demonstrate low densities for high-affinity ^{125}I-pNPY 1–36 binding sites within the hypothalamus and the preoptic area. These results may be related to the possibility (see above) that using receptor autoradiography ^{125}I-NPY 1–36 does not label high-affinity receptors of the Y2 type. To elucidate this possibility further we have in the present experiments studied the actions of centrally administered pNPY 13–36 on the serum levels of hypophyseal hormones in the awake unrestrained male rat.[19] The results are shown in FIGURE 9. With regard to effects on serum CORTICO levels pNPY 13–36 produces a U-shape dose-response curve with a marked and significant reduction of serum CORTICO levels in a dose of 75 pmoles/rat (FIG. 9A). These inhibitory effects are no longer observed after inhibition of tyrosine hydroxylase (TH). The restoration of serum CORTICO levels with the highest dose used of the pNPY fragment may be explained on the basis that the pNPY fragment in the highest dose also activates low-affinity Y2 receptors. These results indicate that high- and low-affinity NPY receptors of the Y2 type may play a role in the control of CORTICO secretion via regulation of corticotropin releasing factor (CRF) released from the median eminence.

With regard to PRL secretion the pNPY fragment 13–36 is shown to reduce serum PRL levels at the 1 h time interval in the doses of 750 and 1250 pmol (FIG. 9B). These inhibitory affects are abolished after TH inhibition. In contrast, the TH inhibitor unmasks marked inhibitory effects of the pNPY fragment in the lowest dose range of 7.5 and 25 pmoles/rat. Thus, both high- and low-affinity Y2 NPY receptors seem to participate in the inhibitory control of PRL secretion. The actions at the high-affinity Y2 receptor is un-

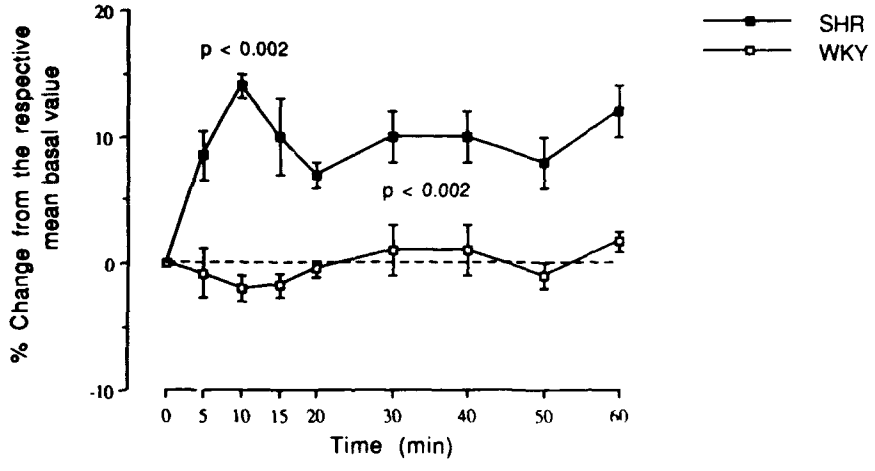

FIGURE 6. Comparison between the time-curve of per cent change of mean arterial blood pressure (MAP) (mm Hg) after an i.v.t. injection of a low dose of pNPY 13–36 (25 pmol) in the spontaneously hypertensive awake freely moving male rat (SHR) and in the normotensive awake freely moving male Wistar-Kyoto control rats (WKY). The Mann-Whitney U test was used to compare the two different groups. The peak ($p < 0.002$) and overall effect ($p < 0.002$) were significantly different. N = 6 in each group. MAP basal values: SHR = 140 ± 2 mm Hg; WKY = 107 ± 4 mm Hg.

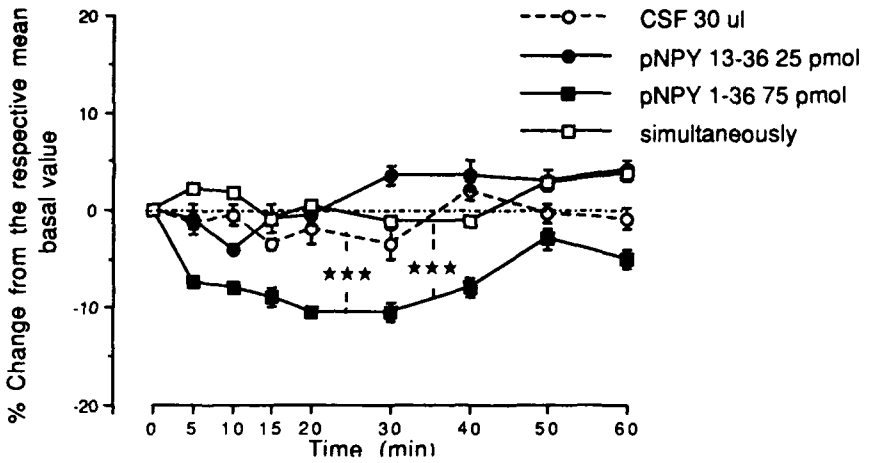

FIGURE 7. Effects on mean arterial blood pressure (MAP) (mm Hg) of i.v.t. pNPY 1–36 at a dose somewhat higher than the ED_{50} value for the peak effect (45 pmol: see Härfstrand, 1987[25]) and of i.v.t. pNPY 13–36 at a threshold dose alone or together in the awake unrestrained male rat. The Dunn's test was used to compare the different groups. The overall effect (the area under the curve) of pNPY 1–36 75 pmol was significantly counteracted by pNPY 13–36 25 pmoles. MAP basal values (mm Hg): CSF = 106 ± 4; pNPY 13–36 25 pmol = 100 ± 5; pNPY 1–36 75 pmol = 98 ± 3; pNPY 1–36 75 pmol + pNPY 13–36 25 pmol = 101 ± 2. *** = $p < 0.005$.

masked by removal of CA. It should be pointed out that the time interval chosen is not optimal for the demonstration of increases in PRL secretion following i.v.t. injection of pNPY 13–36 in view of the fact that i.v.t. pNPY 1–36 mainly increases PRL secretion at the 15 min interval[19] (see below). In previous work it has been possible to demonstrate that pNPY 1–36 upon i.v.t. injection in increasing doses produced a biphasic change in CA utilization in the tuberoinfundibular DA neurons and in the NA utilization in various hypothalamic areas.[56,57] In the low-dose range there is an inhibition of regional hypothalamic CA utilization, while in higher doses an increase of regional hypothalamic CA utilization takes place. A similar analysis has now been performed with the pNPY fragment 13–36 (See FIG. 10). Regional CA levels within the hypothalamus were found to be unaltered (data now shown). In the low dose of 25 pmoles/rat pNPY 13–36 is seen to produce a significant reduction of the CA depletion produced by TH inhibition within the medial palisade zone and the lateral palisade zone of the median eminence (MPZ and LPZ) (FIG. 10). Thus, pNPY 13–36 appears capable of reducing DA utilization both in the MPZ and LPZ. In contrast, in the highest dose used the pNPY fragment produces a significant increase of DA utilization within the median eminence.

These results suggest that both the high- and low-affinity receptors of the Y2 type are involved in the control of the tuberoinfundibular DA neurons and also that the action of pNPY 1–36 on the tuberoinfundibular DA neurons may be mediated via Y2 NPY receptors. It seems likely that at least the increase in DA utilization induced by both pNPY 1–36 and pNPY 13–36 within the MPZ explains the inhibitory changes in PRL secretion demonstrated following i.v.t. injections of pNPY 1–36 and pNPY 13–36.[19,57] Nevertheless, the inhibitory effects observed with pNPY 13–36 in the low doses following TH inhibition must involve a nondopaminergic mechanism.

In conclusion, the NPY regulation of the tuberoinfundibular dopamine (DA) neurons, the PRL secretion and the pituitary-adrenal axis appear to involve both VT, via high-

affinity Y2 receptors, and WT, via low-affinity Y2 receptors. Finally, the strong biological potency of pNPY 13–36 in neuroendocrine control emphasize a role of extracellularly formed C-terminal fragments in NPY transmission within the hypothalamus and thus a dominance of VT.

Angiotensin II-Like Immunoreactivity within Subpopulations of TH Immunoreactive Neurons in the A1 and C1 Area of the Ventral Medulla of the Male Rat

By using double immunolabelling techniques it has recently been possible to demonstrate the coexistence of TH and angiotensin II (ANG II) immunoreactivities within the NA A1 cell group and the adrenaline C1 cell group of the ventral medulla of the rat.[27] Within the A1 area 20–30% of the TH IR nerve cells were ANG II IR and in the C1 area 5–40% of the TH IR nerve cells displayed ANG II IR (FIG. 11). These results open up new possibilities for ANG II/CA/NPY interactions in cardiovascular and neuroendocrine regulation. Thus, the balance of CA NPY and ANG II transmission in these costoring neuronal systems of the ventral medulla may have a decisive influence on the regulation of cardiovascular and neuroendocrine functions. In line with these results it has also been demonstrated that ANG II reduces the affinity of $\alpha 2$ adrenoceptors in membrane preparations from the dorsomedial medulla of the rat.[59]

Angiotensin II/NPY Receptor Interaction

In view of the existence of ANG II/NPY cotransmission in cardiovascular areas binding studies and functional studies have been performed on the potential existence of such

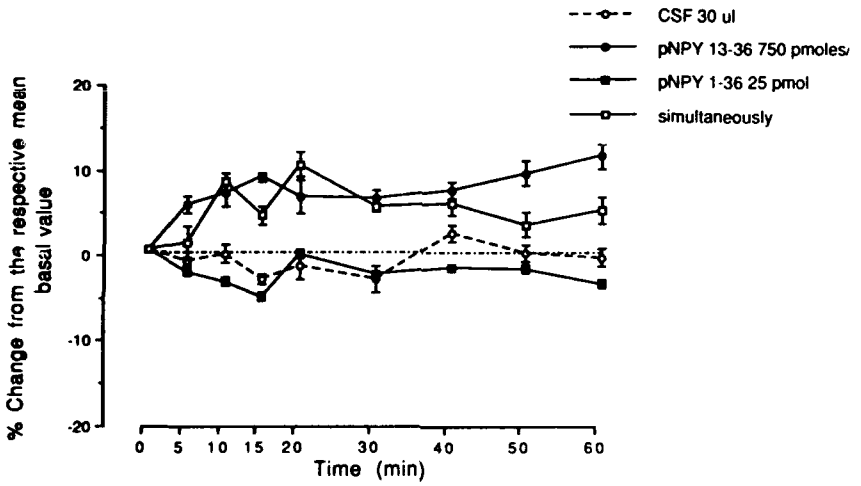

FIGURE 8. Effects of a threshold dose of i.v.t. pNPY 1–36 on the vasopressor effects (MAP; mm Hg) of a submaximal dose of i.v.t. pNPY 13–36 (750 pmol). The Dunn's test was used to compare the different groups: no interaction was found. Thus the pressor effect of the pNPY fragment could not be reduced by a threshold dose of pNPY 1–36. MAP basal values (mm Hg): CSF = 106 ± 4; pNPY 13–36 750 pmol = 109 ± 6; pNPY 1–36 25 pmol = 100 ± 3; pNPY 13–36 750 pmol + pNPY 1–36 25 pmol = 98 ± 3.

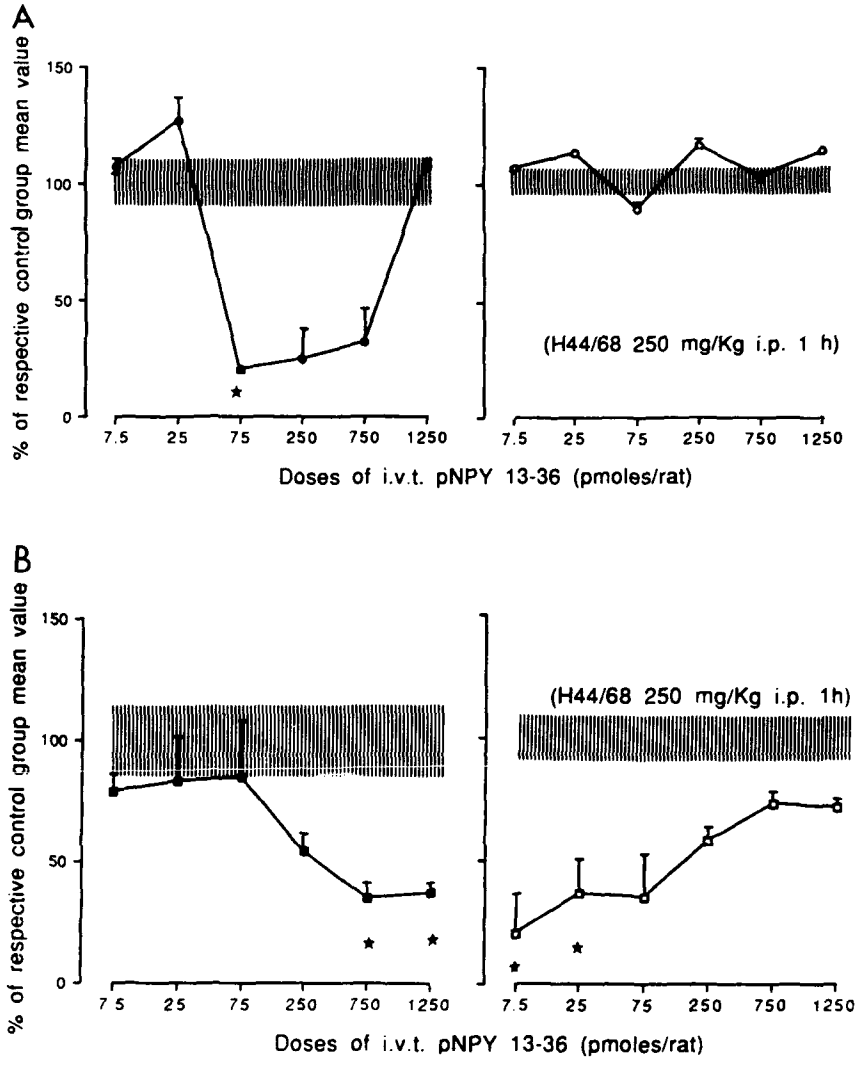

FIGURE 9. *Studies on the effects of six doses of pNPY 13–36 given i.v.t. on serum corticosterone* **(A)** and prolactin **(B)** levels in the absence or presence of tyrosine hydroxylase inhibition (α-MT) in the awake unrestrained male rat. The pNPY fragment (13–36) was given i.v.t. immediately after the i.p. administration of α-MT 1 h before killing. The *striped areas* represent the SEM limits for the respective CSF control groups. The values are given in per cent of the mock CSF alone (CORTICO: 100% = 356 ± 45 nmol/l; PRL: 100% = 4.7 ± 1 ng/ml) and the CSF + α-MT (CORTICO: 100% = 1122 ± 68 nmol/l; PRL: 100% = 66.8 ± 8 ng/ml) treated group mean value. The Treatments *versus* Control nonparametric test was used (★ = $p < 0.05$); n = 6 rats.

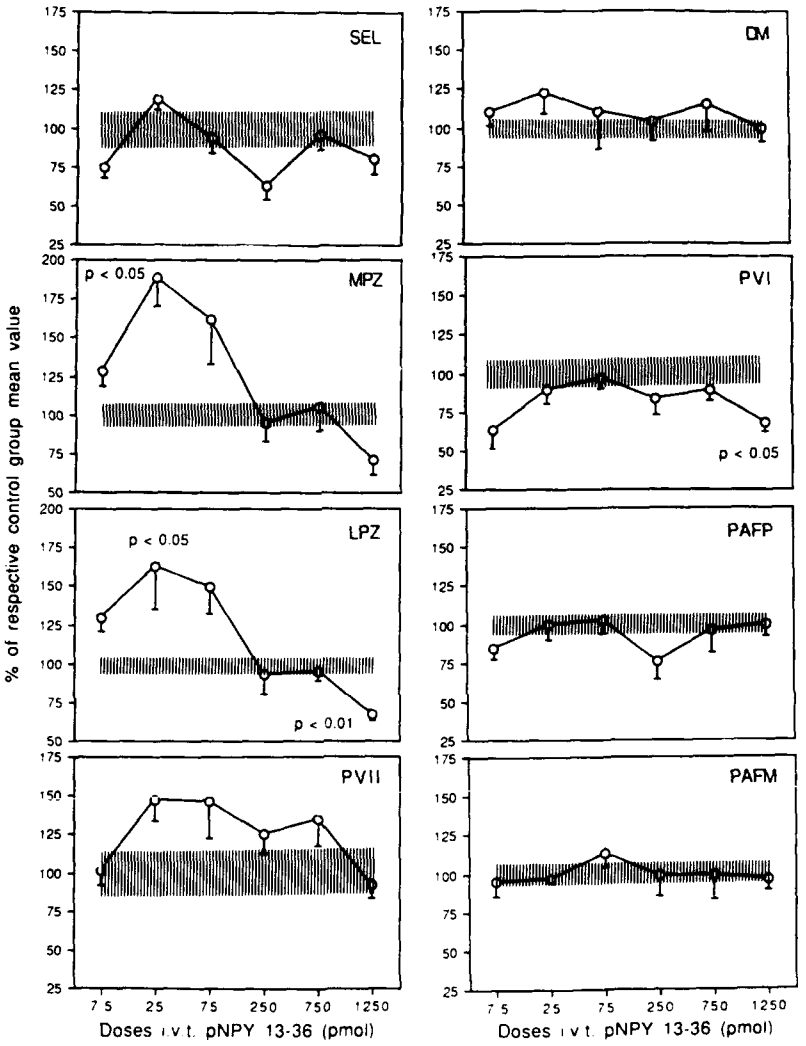

FIGURE 10. Effects of i.v.t. injections of six doses of pNPY 13–36 on CA disappearance in the discrete CA nerve terminal systems of the hypothalamus after TH inhibition (α-MT 250 mg/kg, i.p. 1 h). pNPY 13–36 was given immediately after a-MT treatment. Means ± SEM are shown; n = 6 in each group. The Treatments versus Control nonparametric test was used. The CA levels in the group treated with solvent alone (*shaded area*) were as follows: SEL 100%: 94 ± 4 nmol/g; MPZ 100%: 102 ± 8 nmol/g; LPZ 100%: 277 ± 18 nmol/g; PVII 100%: 40 ± 5 nmol/g; DM 100%: 196 ± 17 nmol/g; PVI 100%: 83 ± 11 nmol/g; PAFP 100%: 228 ± 20 nmol/g; PAFM 100%: 132 ± 11 nmol/g. Abbreviations: SEL, subependymal layer of the median eminence; MPZ, medial palisade zone of the median eminence; LPZ, lateral palisade zone of the median eminence; PVII, posterior periventricular hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus; PVI, anterior periventricular hypothalamic region; PAFP, parvocellular part of the paraventricular hypothalamic nucleus; PAFM, magnocellular part of the paraventricular hypothalamic nucleus.

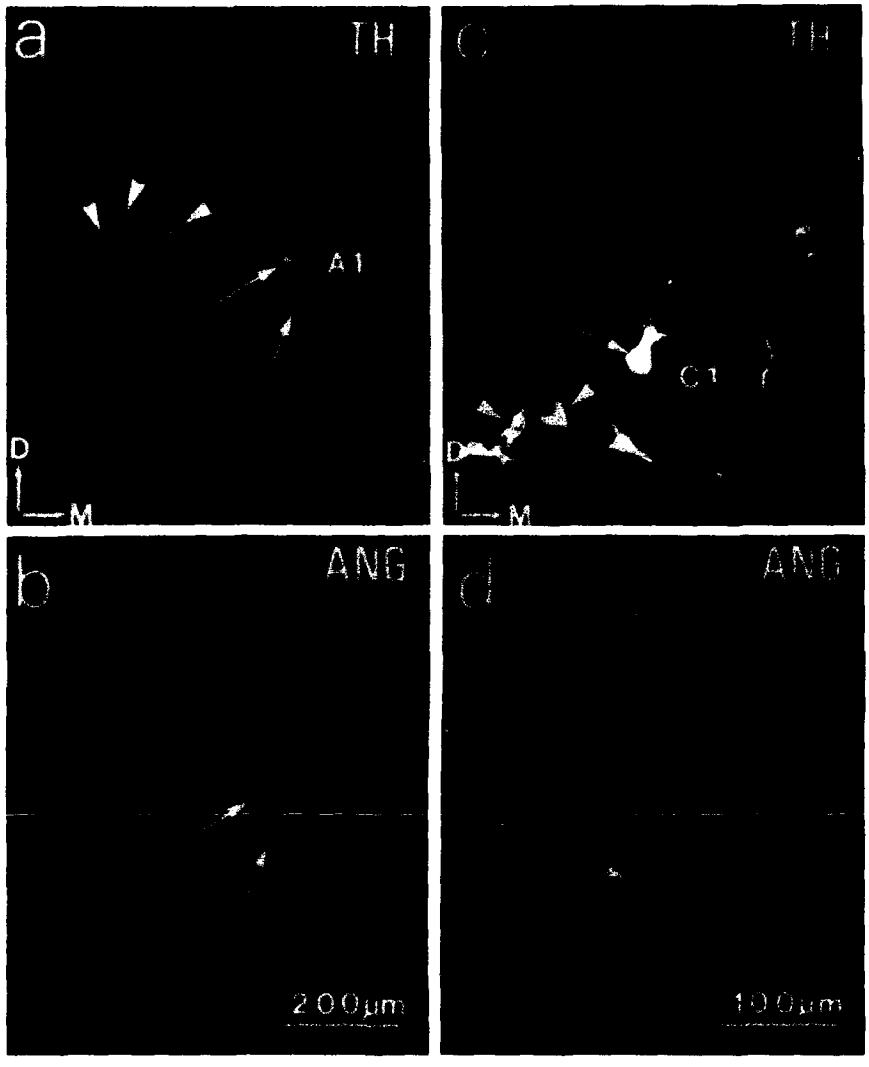

FIGURE 11. Fluorescence microphotographs of tyrosine-hydroxylase (TH) and angiotensin II (ANG II) immunofluorescent (IR) perikarya are shown in the same coronal section in the A1 area (**a,b;** Bregma -14.6 mm) and in the C1 area (**c,d;** Bregma -13.3 mm) of the ventral medulla of the male rat using double immunolabelling procedures. ANG II/TH IR nerve cell bodies are marked by *arrows* and the TH IR nerve cell bodies lacking ANG II immunofluorescence are indicated by *arrowheads*. To facilitate the comparisons of the sections one capillary is marked with an *asterisk*. D, dorsal; M, medial; ANG, ANG II immunofluorescence; TH, tyrosine hydroxylase immunofluorescence. The magnification bars are indicated.

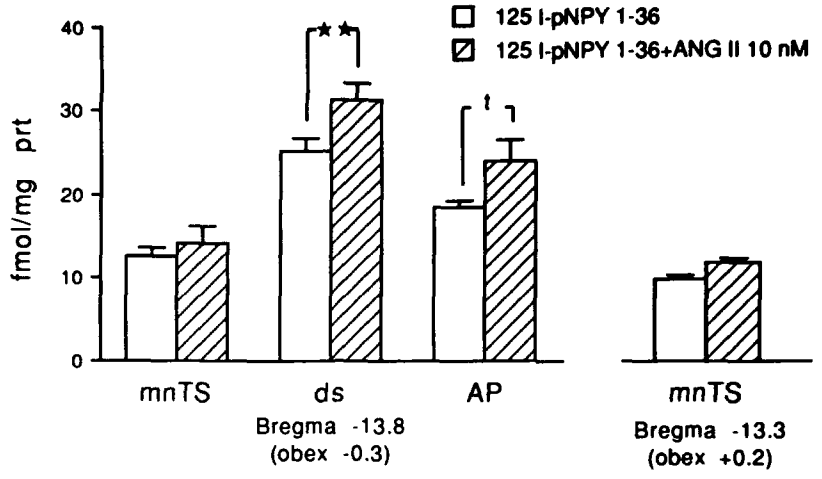

FIGURE 12. Microdensitometric determination of ^{125}I-NPY 1–36 binding in the various regions of the dorsomedial medulla oblongata in the absence or presence of angiotensin II (ANG II; 10 nM) in the male rat at two rostrocaudal levels. For details see Härfstrand 1986.[41] Means ± SEM are shown; n = 4 rats; 0.5 nM of ^{125}I-NPY 1–36 was used. Size of areas measured: nucleus medial subnucleus of tractus solitarius (mnTS) = 0.03 ± 0.007 mm2; dorsal strip (ds) = 0.03 ± 0.002 mm2; area prostrema (AP) = 0.09 ± 0.008 mm2. The Student *t* test was used (** = $p < 0.01$; t = trend).

interactions. In FIGURE 12, it is shown in receptor autoradiographical experiments that *in vitro* incubation with ANG II (10 nM) leads to a significant increase in the ^{125}I-NPY 1–36 binding within the dorsal strip (dorsal cardiovascular region of nTS) and a trend for an increase of the ^{125}I-NPY 1–36 binding in the area postrema. The concentration of ^{125}I-NPY 1–36 was 0.5 nM. In FIGURE 13 it is demonstrated in cardiovascular experiments that a threshold dose of ANG II given i.c. (3 nmoles/rat) not only counteracts the vasodepressor action of ED_{50} dose of pNPY 1–36, but also leads to the development of a highly significant vasopressor actions of ANG II compared with the mock CSF group (FIG. 13). Taken together, the results from the two experiments can be explained on the basis that ANG II receptor activation leads to an uncoupling of the NPY Y1 receptors to its G_i-protein, leading to an increase in the ^{125}I-NPY 1–36 binding (Y1 receptor binding)[47] and thus to a counteraction of the vasodepressor response to pNPY 1–36. The vasopressor action of pNPY 1–36 is then explained by the selective activation at NPY receptors of the Y2 type, which induce vasopressor actions.[55] The ANG II receptor activation may contribute to this pressor action also by enhancing sensitivity at Y2 receptors.

On the Adrenocortical Hormone Regulation of CA/NPY Costoring Neurons

It has recently been possible to demonstrate the existence of strong nuclear glucocorticoid receptor (GR) IR within all the NA/NPY and adrenaline/NPY neurons of the brain stem of the rat.[60–62] It is of substantial interest that these cardiovascular neurons contain high amounts of GR in view of the participation of GR in the regulation of blood pressure.[63] Furthermore, adrenaline and NPY synthesis as well as adrenaline/NPY costorage appear to be controlled by GR,[64–66] suggesting one mechanism for the ability of GR to influence central cardiovascular control. As seen in FIGURES 3 and 14, adrenalec-

tomy appears to differentially regulate the vasodepressor responses induced by pNPY and adrenaline in the awake unrestrained male rat[28] (FIG. 3). Thus, adrenalectomy alone significantly reduced the vasodepressor responses to i.v.t. adrenaline but not to i.v.t. pNPY. Thus, after adrenalectomy the central vasodepressor responses of pNPY dominate, since the adrenergic vasodepressor responses are selectively reduced. Furthermore, corticosterone treatment abolished the centrally evoked vasodepressor responses to close to maximal doses of adrenaline and pNPY, which may contribute to the hypertensive properties in man. Thus, it seems possible that when the pituitary-adrenal activity is substantially reduced the NPY transmission dominates over adrenaline transmission within the cardiovascular CA/NPY neurons. In line with these cardiovascular results it has recently been shown that adrenalectomy leads to an increase in ^{125}I-NPY 1–36 binding within the area postrema and the nTS, an increase which is abolished by corticosterone treatment.[65]

CONCLUSION

The present findings give further evidence for an important role of NPY in central cardiovascular regulation and also emphasizes the importance of the C-terminal pNPY fragment 13–36 in cardiovascular regulation, by selectively acting on NPY receptors of the Y2 type. In contrast to pNPY 1–36 which produces marked vasodepressor actions by activation of NPY receptors of the Y1 type, the pNPY fragment 13–36 produces vaso-

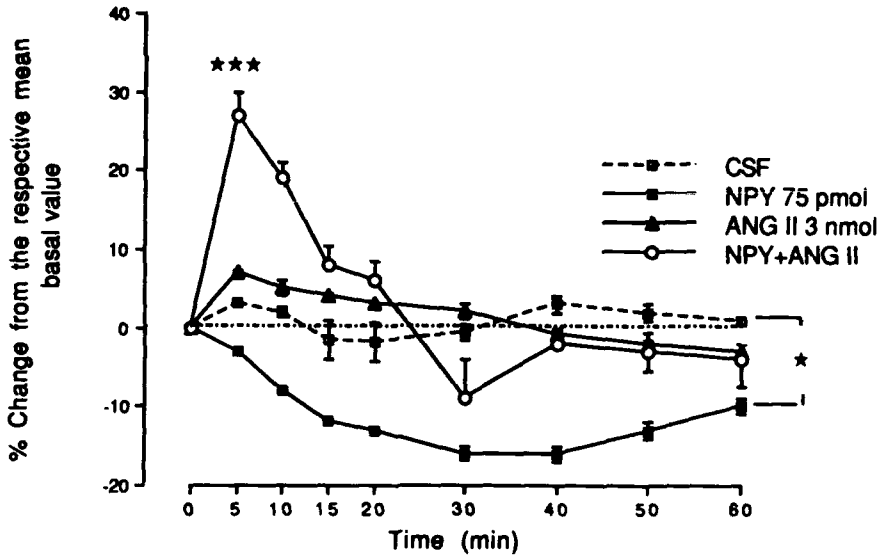

FIGURE 13. Effects on mean arterial blood pressure (MAP) (mm Hg) by i.v.t. pNPY 1–36 at a dose (75 pmol) somewhat higher than the ED_{50} value for the peak effect (45 pmol; see Härfstrand, 1987[25]) and interactions with a threshold dose of angiotensin II (ANG II) in the α-chloralose (100 mg/kg) anaesthetized male rat. The Dunn's test was used to compare different groups (* = $p < 0.05$ for the peak effect between the pNPY 1–36 alone group and the other three groups; *** = $p < 0.005$ for the overall effect, between vasodepressor area for mock CSF and vasodepressor area for pNPY 75 pmol). MAP basal values (mm Hg) for each group (n = 4): CSF = 106 ± 5; pNPY 75 pmol = 110 ± 6; ANG II 3 nmol = 122 ± 2; pNPY 75 pmol + ANG II 3 nmol = 100 ± 5.

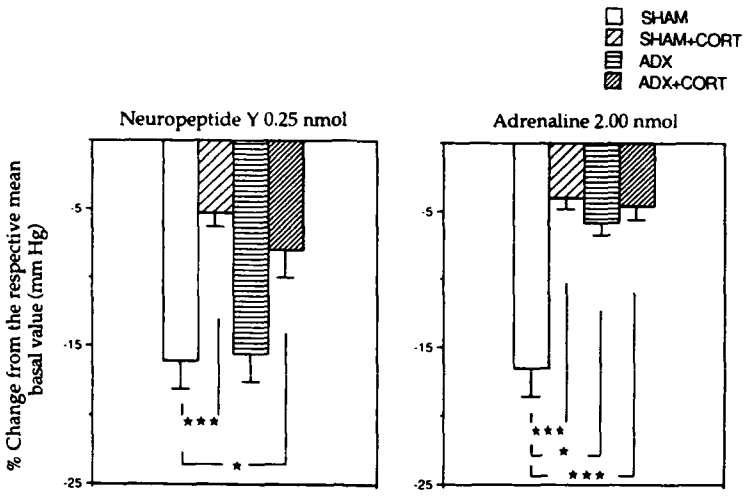

FIGURE 14. Effects of changes in pituitary-adrenal activity on the overall vasodepressor effects (area values) induced by porcine neuropeptide Y (pNPY) 0.25 nmol and adrenaline (A) 2.00 nmol given intraventricularly in the same rats as reported in FIGURE 1. The effects of mock CSF (sham + veh = 5.3% ± 0.6; sham + cort = 5.7% ± 3; adx + veh = 8% ± 2; adx + cort = 8.5% ± 1) have been taken away from the cardiovascular values obtained with pNPY and A. Data are given as means ± SEM. Area values are expressed in arbitrary units. Dunn's test for multiple comparisons: ⋆ = $p < 0.05$; ⋆⋆ = $p < 0.01$; ⋆⋆⋆ = $p < 0.005$.

pressor responses via selective activation of the high-affinity NPY receptors of the Y2 type. Thus, chemical networks appear to exist within NPY transmission involved in cardiovascular regulation, leading to the production of syndromic responses and feedback actions on the Y1 transmission line. The studies on the pNPY fragment 13–36 in combination with the demonstration of morphological and functional transmitter-receptor mismatches in the central cardiovascular NPY neurons suggest a role of VT in the NPY transmission in cardiovascular regions. Available data also underline a potential role of peptidases and peptidase inhibitors in NPY transmission in cardiovascular control. This regulation may be altered in SHR in view of the enhanced pressor action by pNPY 13–36 in this strain of rats. Finally, a nerve impulse-regulated switch between transmission lines in the adrenaline/NPY neurons appear to underlie the duration of the biological action[14,26] and the degradation-regulated switch between isoreceptor transmission lines may underlie the direction of the cardiovascular action of pNPY 1–36. These switching phenomena may be facilitated by intramembrane receptor-receptor interactions such as those between NPY Y1 and α2 receptors, NPY Y1 and ANG II receptors, and NPY isoreceptors.

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Neuropeptide Tyrosine in the Cardiovascular System[a]

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INTRODUCTION

Neuropeptide tyrosine (NPY) is an amidated 36-amino acid sequence, produced by posttranslational processing from a 97-amino acid precursor molecule (preproNPY), and is one of the most abundant neuropeptides identified in the mammalian nervous system. Removal of the 28-amino acid N-terminal signal peptide gives rise to proNPY which undergoes proteolytic processing and amidation to generate both NPY_{1-36} and a 30-amino acid C-terminal flanking sequence ($proNPY_{40-69}$ or C-PON).[1-3] NPY was originally isolated from the pig brain, but has since been identified in tissues from several other mammals (man, rat, rabbit, guinea pig, ox and sheep) and found to be a highly conserved sequence,[4] which is widely distributed in the peripheral nervous system.

Immunohistochemistry

We have used specific antisera raised against both synthetic human NPY and C-PON sequences to investigate the distribution of NPY-containing nerves in the mammalian cardiovascular system. C-PON immunoreactivity occurs naturally in mammalian tissues[5] and has an identical distribution pattern to that of NPY immunoreactivity,[6] both sequences being co-localised at the light and ultrastructural level in human cardiac nerve terminals.[7-8] Optimum immunostaining of NPY- and C-PON-containing nerves has been obtained using tissue fixed by immersion, for up to 48 hours at 4°C, in a modified Bouin's solution[9] containing 85 ml of 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) and 15 ml of saturated picric acid per 100 ml of fixative. After fixation, tissues are rinsed for 24–48 hours in several changes of phosphate buffer, containing 15% (w/v) sucrose and 0.1% sodium azide, and processed immediately as either whole mount preparations or cryostat sections.[7,8,10] Prior to immunostaining, the tissues are incubated in phosphate buffered saline containing 0.2% Triton X-100 for 30–120 minutes and counterstained with Pontamine Sky Blue, to aid antibody penetration and reduce background autofluorescence respectively.[11]

Peripheral nerves are readily visualised using indirect immunofluorescence techniques and the general distribution pattern of cardiovascular innervation may be demonstrated using antisera to neural marker proteins such as protein gene product 9.5 (PGP 9.5), synaptophysin and the Schwann cell marker S-100.[7,8,10,12] PGP 9.5 is a human neuron-specific cytoplasmic protein which has recently been found to correspond to a ubiquitin

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carboxyl-terminal hydrolase,[13] and synaptophysin is an integral membrane protein of secretory vesicles in nerve terminals which contain the classical neurotransmitters acetylcholine and noradrenaline.[14] Polyclonal antiserum to PGP 9.5 has been found to immunostain autonomic and sensory nerves, from the cell body to distal varicose nerve terminals, in peripheral tissues of man as well as other species (*e.g.*, guinea pig). Synaptophysin immunoreactivity on the other hand is localised specifically to varicose nerve terminals and appears to exhibit a more extensive distribution in the peripheral nervous system of certain animals (*e.g.*, rat and cow) than does PGP 9.5. Subpopulations of cardiac nerves may be identified according to their neuropeptide and enzyme content, relative distribution, axon ultrastructure and sensitivity to selective neurotoxins.

NPY-Immunoreactive Cardiac Nerves

Nerve fibres displaying NPY and C-PON immunoreactivity are widely distributed in the mammalian heart where they are concentrated in the myocardium, endocardium, intracardiac ganglia and in association with the coronary vasculature. While relatively few immunoreactive fibres occur around epicardial blood vessels in the human heart a dense perivascular network supplies small intramyocardial arteries and arterioles (Fig. 1), forming a plexus of varicose fibres and fascicles at the adventitial-medial border. The myocardium receives fewer nerves than intramyocardial vessels, but is also well supplied with varicose fibres containing NPY and C-PON. Immunoreactive nerve fibres occur throughout the walls of all the heart chambers, however, a density gradient exists between the atria and ventricles. NPY-immunoreactive nerves are also found in the cardiac conduction system and in the cow, for example, they occur distributed in sinus and atrioventricular nodes (Fig. 2) as well as in ventricular bundle branches.[15] The density of the sinus and atrioventricular node innervation as a whole is significantly greater than that observed elsewhere in the mammalian heart, whereas the density of NPY-immunoreactive nerves is generally comparable to that found in the adjacent myocardium. In the conduction system of the guinea pig (Fig. 3) and human heart, for example, NPY-immunoreactive nerves represent a relatively minor subpopulation compared to the numerous nerve fibres displaying PGP 9.5 and synaptophysin immunoreactivity, as well as acetylcholinesterase activity. Intracardiac ganglia in the atrial subendocardium, atrioventricular groove and in the region of the sinus node contain NPY/C-PON-immunoreactive nerve fibres, but intrinsic neuronal cell bodies appear to lack immunoreactivity in the human heart.[10] In the human fetal heart, NPY/C-PON-immunoreactive nerves are detected after 10 weeks of gestation and occur first in the region of the sinus and atrioventricular nodes and atrial epicardium. The ventricular innervation develops later and accompanies the coronary vasculature, with prominent networks of fine varicose fibres distributed around epicardial vessels and extending together with arterial branches into the myocardium (Fig. 4). Although still comparatively few in number, the general distribution of NPY-immunoreactive cardiac nerves at 21–24 weeks of gestation appears to be similar to that observed after birth, and as in the adult, these nerves represent the predominant peptide-containing nerve population detected in the fetal heart.

Posttranslational processing and amidation of NPY are essential for NPY receptor binding and activity,[16] and recent immunohistochemical findings indicate that NPY present in human cardiac nerve fibres occurs in an amidated form.[17] Immunohistochemical studies have revealed that NPY-immunoreactive nerves generally exhibit a similar distribution pattern to that of nerves containing immunoreactivity for two enzymes involved in the catecholamine synthetic pathway, tyrosine hydroxylase and dopamine beta-hydroxylase (Fig. 5), suggesting that NPY-immunoreactive cardiovascular nerves are of sympathetic origin.

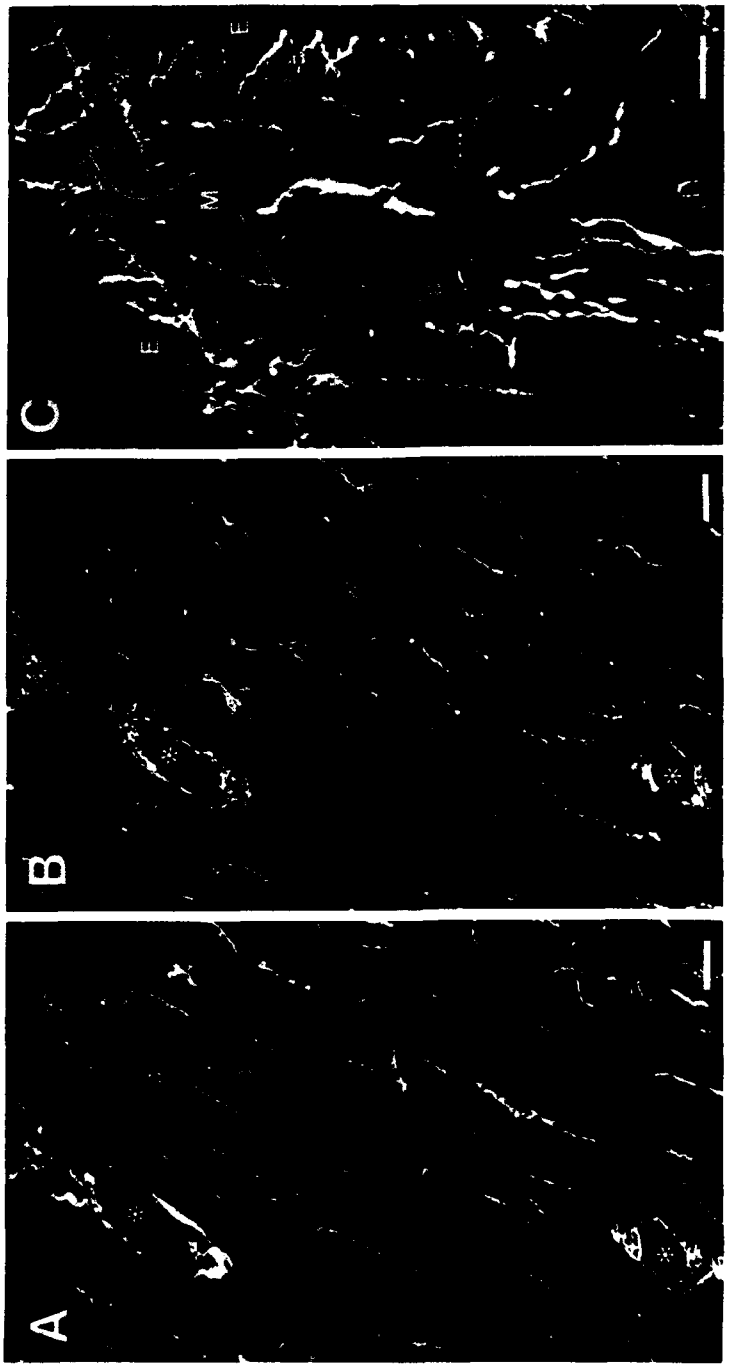

FIGURE 1. Immunofluorescence micrographs showing nerve fibres and fascicles displaying PGP 9.5 (**A**) and C-PON immunoreactivity (**B**) in serial sections of human right atrium. Nerve fibres are concentrated in the perivascular plexus around intramyocardial arteries and arterioles (*asterisk*) and distributed among myocardial fibres. A dense network of C-PON-immunoreactive nerve fibres also occurs in the subendocardial plexus of the right atrial wall (**C**). E, endocardium; M, myocardium. Bar = 50 μm.

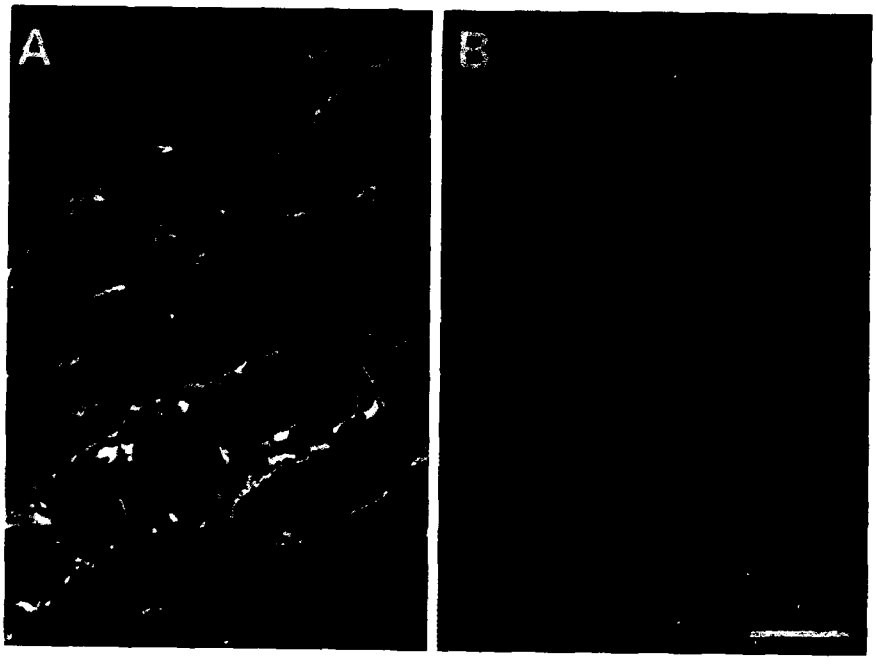

FIGURE 2. Immunofluorescence micrographs showing synaptophysin-immunoreactive nerve fibres concentrated around conduction cells in the atrioventricular node of the bovine heart **(A)**. A subpopulation of fine varicose nerve fibres display NPY immunoreactivity **(B)**. Bar = 50 μm.

Origins of NPY-Immunoreactive Cardiac Nerves

The presence of NPY immunoreactivity in sympathetic cardiovascular nerves has been further supported by the results of both surgical and chemical denervation studies. During human heart and heart-lung transplantation the extrinsic efferent and afferent nerve supply to the donor heart is sectioned and the available functional and anatomical evidence suggests that there is no significant reinnervation of the allograft.[10] Nerve fibres, fascicles and ganglia are present in human cardiac allografts, at least up to 7–8 years after transplantation, and display immunoreactivity for PGP 9.5, synaptophysin and S-100 as well as acetylcholinesterase activity. Compared with innervated donor and explanted recipient or postmortem hearts there is, however, a lack of NPY/C-PON- and tyrosine hydroxylase-immunoreactive nerves in allograft tissues, suggesting that they are of extrinsic origin. These observations are consistent with the results of surgical and chemical sympathetic denervation studies in experimental animals which result in a 50–90% reduction in NPY and noradrenaline levels.[18–23] Most NPY-containing mammalian cardiac nerves are therefore likely to represent postganglionic sympathetic nerves originating in the stellate and other paravertebral ganglia.[18,19,24] The extent to which sympathectomy depletes cardiac NPY levels varies between species and may reflect differences in the relative contribution of intrinsic neurons to the NPY-immunoreactive innervation of the heart. As in other studies,[24] we have been unable to demonstrate the presence of NPY- or C-PON-immunoreactive neurons in the human heart, but immunohistochemical investigations have identified subpopulations of immunoreactive cardiac neurons in tissue sec-

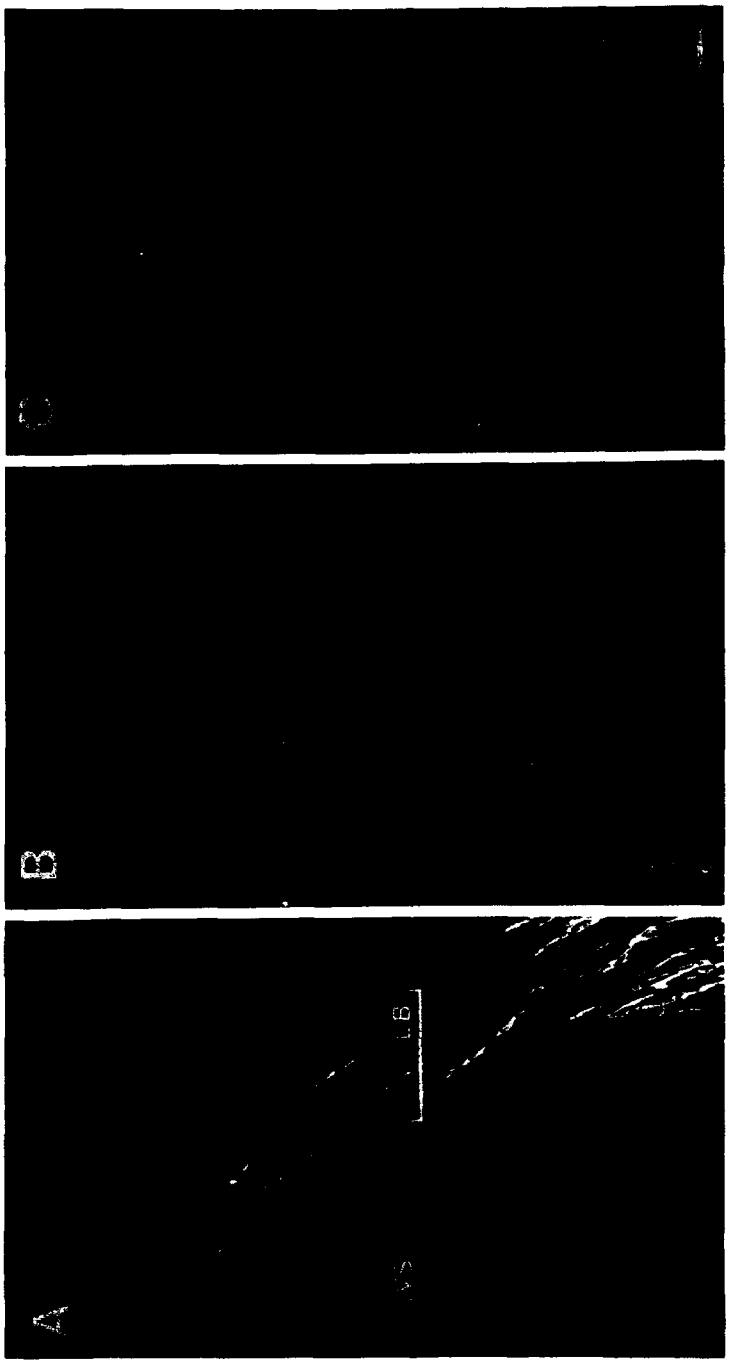

FIGURE 3. Immunofluorescence micrographs of adjacent sections through the interventricular septum of the guinea pig heart, showing nerve fibres in the left bundle (LB) of the ventricular conduction system and in the underlying myocardium. Numerous synaptophysin-immunoreactive nerves are present in the bundle (**A**). Subpopulations of nerves, with distinct distribution patterns, exhibit C-PON (**B**) and calcitonin gene-related peptide immunoreactivity (**C**). Bar = 50 μm.

FIGURE 4. Section of human fetal heart, at 17 weeks of gestation, displaying a dense plexus of NPY immunofluorescent nerve fibres associated with an epicardial coronary artery (CA) and scattered in the underlying ventricular myocardium. Bar = 50 μm.

tions and cell cultures from other mammals.[15,21,25–28] In contrast to the human heart, intrinsic cardiac neurons appear to make a significant contribution to the NPY-immunoreactive innervation of the rat heart,[23] in which no significant reduction of NPY-immunostained nerves was found following heterologous cardiac transplantation into the abdominal cavity.[29] NPY mRNA has also been detected in extracts of rodent atria and ventricles, but the neural origin of this expression is uncertain,[30–31] and it is known that non-neuronal cells in the rat also contain NPY mRNA.[32] In addition to NPY, some mammalian cardiac neurons have been reported to contain other neuropeptides,[24,33] suggesting that the intrinsic cardiac innervation is more complex than was previously thought and does not simply represent a local postganglionic parasympathetic nervous system.

Ultrastructural Localisation of NPY Immunoreactivity

Electron microscopic examination of human cardiac tissue has demonstrated the presence of NPY and C-PON immunoreactivity in axon profiles displaying the ultrastructural features of presumptive sympathetic nerve terminals, immunoreactivity being localised to large granular vesicles (70–100nm diameter) rather than the numerous small vesicles (40–60nm diameter) which occur in immunostained axonal varicosities.[7–8] The results of subcellular fractionation studies also indicate that while noradrenaline is present in both the small and large vesicle populations, NPY immunoreactivity is concentrated in the latter.[34–35] Electrical stimulation of cardiac sympathetic nerves is associated with an

overflow of NPY from the pig heart *in vivo*,[36] and sympathetic activation in man is accompanied by an increase in the plasma concentrations of noradrenaline and NPY, suggesting a co-release of both substances from stimulated sympathetic nerve terminals.[37–38] Other studies have demonstrated the exocytotic release of both NPY and noradrenaline in the pig spleen[39] and guinea pig heart,[40] and the distinct subcellular localisation of NPY may facilitate the differential release of NPY and noradrenaline, high frequency stimulation resulting in the preferential release of NPY.[39]

Pulmonary NPY-Immunoreactive Nerves

As in the heart and systemic blood vessels, NPY- and C-PON-immunoreactive nerves are the predominant peptide-containing nerve population identified in the pulmonary vasculature[41] and airways[42] of human infants and children. In the lungs of patients

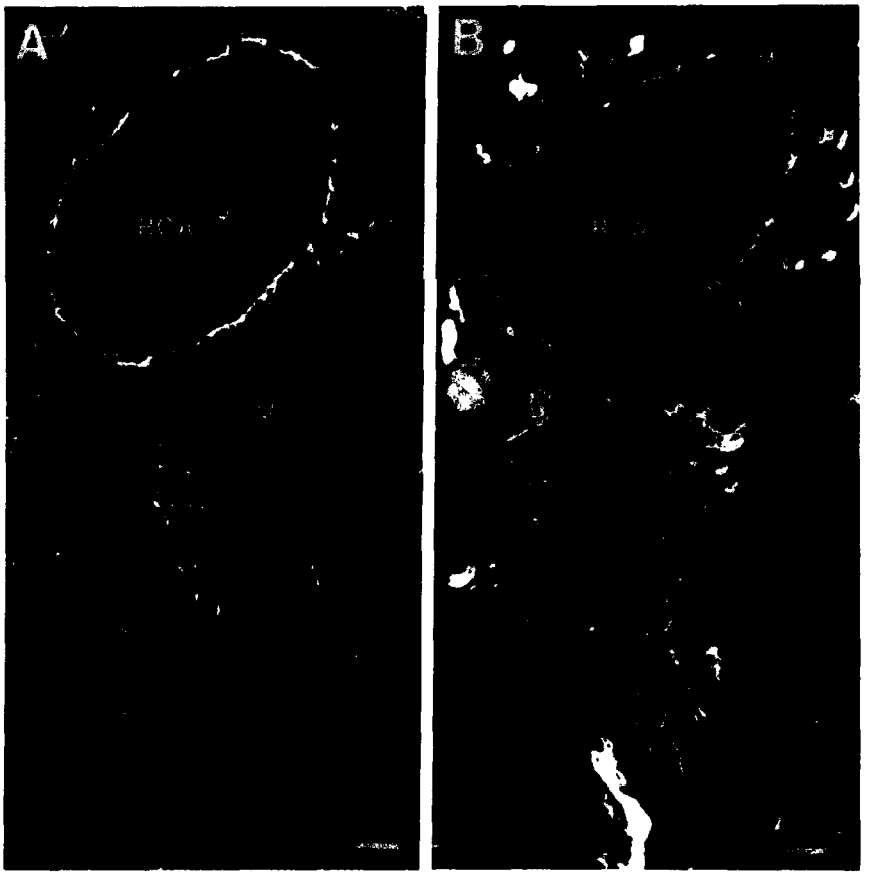

FIGURE 5. Immunofluorescence micrographs of serial sections of the right coronary artery (RCA) in the guinea pig heart showing the similar distribution pattern of NPY- **(A)** and tyrosine hydroxylase-immunoreactive **(B)** nerve fibres and fascicles. Bar = 50 μm.

without cardiopulmonary disease, aged 1 month to 24 years, NPY-immunoreactive nerve fibres and fascicles accompany intrapulmonary arteries and veins to the level of the respiratory unit, but were not observed associated with vessels in the alveolar wall. Proximal elastic intrapulmonary arteries are accompanied by nerve trunks and scattered fibres, the perivascular plexus being denser around smaller muscular arteries where nerve fibres and fascicles run along and around the vessels in the adventitial-medial border. Unlike developing porcine pulmonary arteries,[43] NPY-immunoreactive nerves were not seen in the media of human muscular arteries and only rarely penetrated into the outermost lamellar unit of elastic arteries. NPY-containing nerves were prominent in the myocardial sheath surrounding extrapulmonary veins and in contrast to the arterial innervation, nerve fibres penetrate the muscle coat of intrapulmonary veins.[41] Age-related changes appear to be confined to the respiratory unit in the human lung, with the density of alveolar duct artery innervation increasing from 1 month to 2.5 years. Differences in patients with pulmonary hypertension also appear to be confined to the respiratory unit rather than in proximal regions, pulmonary hypertension being associated with a premature innervation of arteries in the respiratory unit by NPY-immunoreactive nerves (Fig. 6). NPY nerves are also the most common peptide-containing subpopulation identified in developing human airways, supplying bronchial and bronchiolar smooth muscle, submucosal glands, cartilage and submucosa.[42] Within the respiratory unit, airway innervation is less dense than that in accompanying pulmonary arteries and while NPY/C-PON-immunoreactive nerves extend into the respiratory bronchiole they were not seen in the alveolar duct region.

Functional Significance

It is uncertain to what extent the relative density of NPY-immunoreactive innervation in the heart vasculature and lung relates to the reactivity of these tissues to NPY. The presence of a dense perivascular nerve plexus around human intramyocardial coronary arteries is, however, consistent with the results of functional studies which indicate a predominant vasomotor role for NPY in the cardiovascular system, causing vasoconstriction of small resistance vessels rather than large epicardial arteries in the human heart.[44] The effects of NPY vary in different vascular beds, but it is now recognised that as well as exerting a direct vasoconstrictor effect NPY can also influence blood flow by pre- and postjunctional effects, reducing the release of noradrenaline and potentiating the responses induced by other vasoconstrictor agents.[45–47] The functional significance of the numerous NPY-immunoreactive nerve fibers present in the myocardium and subendocardium has still to be established since NPY seems to have no direct effect on human myocardial contractility.[48–49] NPY may, however, modulate autonomic activity in the heart and attenuate vagal effects on the atrial myocardium and conduction system, acting presynaptically to inhibit the release of acetylcholine from autonomic nerve terminals.[50] Whether age-related differences in human pulmonary and airway innervation are of functional significance is not known, but the predominant innervation of small, abnormally thick-walled precapillary vessels by nerves containing vasoactive NPY may contribute to the susceptibility of infants to pulmonary hypertensive crises.[41]

In conclusion, NPY-immunoreactive nerves are widely distributed in the cardiovascular system of man and other mammals and are the predominant peptide-containing nerve population supplying the human heart and vasculature. There is now substantial evidence indicating that cardiovascular NPY-immunoreactive innervation is mainly of sympathetic origin, although in some species intrinsic cardiac neurons may also represent a further significant source of NPY-containing cardiac nerves. Functional studies have implicated NPY in the regulation of vascular function, but its significance in the heart and lung is still not fully understood.

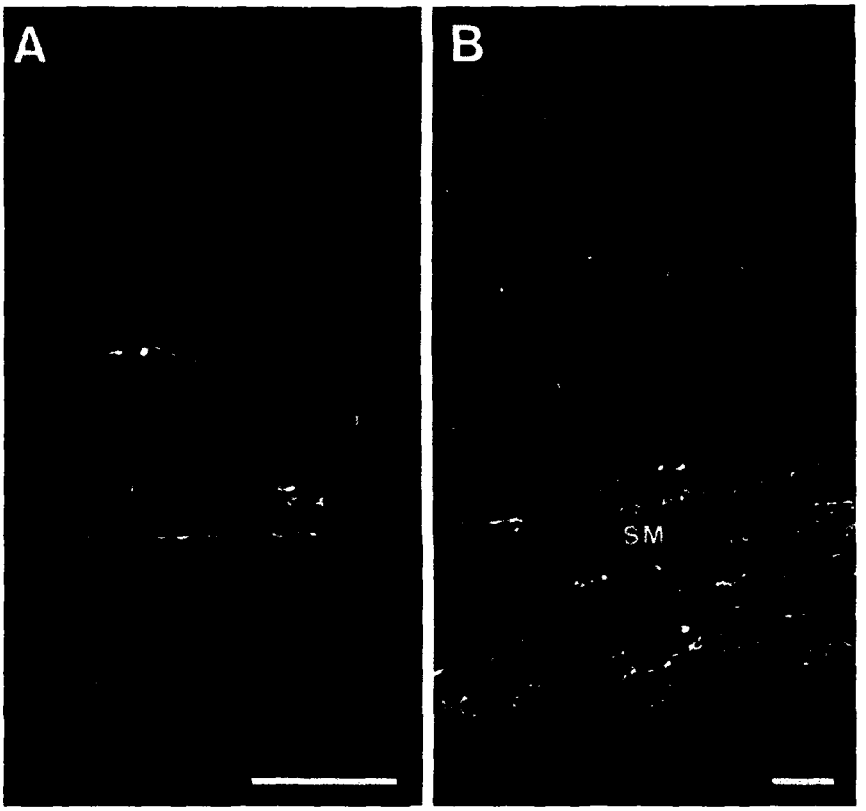

FIGURE 6. Immunofluorescence micrographs showing NPY-immunoreactive nerve fibres associated with an abnormally thick-walled intra-acinar artery in a 2-month old child with pulmonary hypertension **(A)** and in the bronchial smooth muscle (SM) of a child at 10 years of age **(B)**. Bar = 50 μm.

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In Vitro Effects of Neuropeptide Y at the Vascular Neuroeffector Junction[a]

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Neuropeptide Y is a 36 amino acid peptide that is one of the most abundant neuropeptides within the central and peripheral nervous systems.[1-3] In the periphery, NPY is present in many noradrenergic sympathetic neurons and is particularly dense in blood vessels.[4-6] The finding of NPY in noradrenergic neurons in blood vessels obviously adds NPY to the list of peptides exerting important actions on the cardiovascular system. The purpose of this paper is to summarize the results of recent studies from our laboratory where we have examined the effect of NPY on a model vascular neuroeffector junction—the perfused mesenteric arterial bed of the rat.

METHODS

Experiments were carried out in various strains of rats including spontaneously hypertensive (SHR), Wistar Kyoto (WKY) (Taconic Farms, Germantown, NY) and Sprague Dawley (SD) (Sasco, St. Louis, MO). Animals were maintained under standard laboratory conditions with food and water ad libitum. Blood pressure was monitored in conscious rats by tail cuff plethysmography one or two days prior to experimentation.

The perfused mesenteric arterial bed was utilized as described by us previously.[7] Briefly, the mesenteric vascular bed was dissected from the rat, perfused (5 ml/minute) and superfused (0.5 ml/minute) with modified Krebs bicarbonate buffer bubbled with 95% O_2 and 5% CO_2 at 37°C. A pair of platinum electrodes were placed around the mesenteric artery for periarterial nerve stimulation (various frequencies, 2 ms duration supramaximal voltage). The preparation was allowed to equilibrate for 45–60 minutes prior to experimentation. Perfusion pressure was continuously monitored by pressure transducers coupled to chart recorders. In some experiments perfusate effluents were collected in 1-minute fractions into tubes containing 0.1 N perchloric acid, concentrated by alumina chromatography, separated by high performance liquid chromatography (HPLC) and quantified by electrochemical detection.

To examine the concept that there are subtypes of NPY receptors, a series of C-terminal fragments were generously provided by Dr. Jean Rivier of the Salk Institute. The fragments were examined for pre- and postjunctional actions in the perfused mesentery as well as their ability to produce depressor effects following intrathecal administration and to inhibit the K^+-induced release of norepinephrine from brain slices. Fragments studied

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included NPY 11–36, 14–36, 16–36, 17–36 and 18–36. Additional peptides studied included NPY 1–36, PYY 1–36 and PPY 1–36.

The effect of NPY and NPY fragments on the potassium (K^+) induced release of NE from slices of the posterior hypothalamus (PH) or anterior hypothalamus (AH) was investigated as described by us previously.[8] K^+ (56 mM) was used during two stimulation periods (S_1 and S_2) to evoke the release of NE measured by HPLC-EC detection or by liquid scintillation spectrometry from slices of the hypothalamus obtained from SD, SHR, or WKY. Drugs were added prior to S_2. Other experiments examined the effect of drugs on the release of NPY-like immunoreactivity (NPY-LI) from brain slices and measures by a specific radioimmunoassay.[9]

For intrathecal injections (Int), animals were prepared according to the method of Yaksh & Rudy[10] and described by us in detail previously.[11] Briefly the atlantooccipital membrane was exposed by midline incision through the trapezoid and rhomboid muscles. Keeping the head flexed at 35°, one polyethylene catheter (PE-10) was inserted down the lower thoracic spinal subarachnoid space (T_{10}) through a puncture of the atlantooccipital membrane. Drugs were dissolved in saline and slowly injected (over 20–30 sec) at a volume of 5 μl injection of vehicle to wash the catheter.

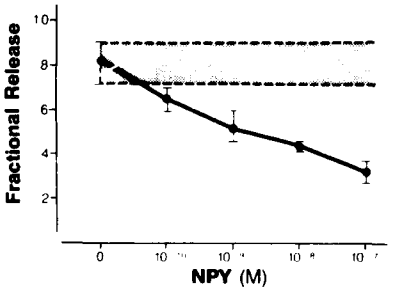

FIGURE 1. The effect of NPY on the periarterial nerve stimulation-induced release of endogengeous NE from the perfused mesenteric artery of the rat. Data are plotted as the fractional release of endogenous NE verses the concentration of NPY. Each point is the mean ± SEM of at least 6 observations. NPY produced a concentration-induced inhibition of the evoked release of NE.

RESULTS

Pre- and Postjunctional Effects of NPY in the Mesenteric Arterial Bed

NPY produced both pre- and postjunctional effects on noradrenergic transmission in the perfused mesenteric artery of the rat.[5,12] The peptide exerted a concentration-dependent decrease in the periarterial nerve stimulation-induced release of endogenous NE (FIG. 1) The threshold concentration of NPY to exert this inhibitory effect was approximately 10^{-10} M. At concentrations of 10^{-10} and 10^{-9} M the inhibitory effect of NPY on NE release resulted in a small attenuation of the increase in perfusion pressure resulting from periarterial nerve stimulation.[5] However starting at 10^{-8} M and higher, NPY produced a potentiation of the increase in perfusion pressure. A representative tracing of the NPY-induced potentiation of nerve stimulation induced increase in perfusion pressure is depicted in FIGURE 2. The potentiation of the increase in perfusion pressure to periarterial nerve stimulation also seen when perfusion pressure was increased by other vasoactive agents. FIGURE 3 depicts the effects of NPY on the increase in perfusion pressure produced by arginine vasopressin. Similar potentiation was seen when perfusion pressure was increased by angiotensin,[11] as well as phenylephrine.[12]

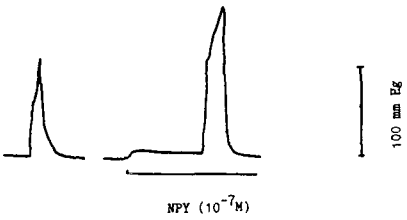

FIGURE 2. The periarteriatial nerve stimulative (16 Hz, supramaximal voltage) induced increase in perfusion pressure (mmHg) alone (*first tracing*) or in the presence of 10^{-7} M NPY (*second tracing*). NPY produced a potentiation of the periarterial nerve stimulation increase in perfusion pressure.

The prejunctional inhibitory effect of NPY was not altered by α_1 or α_2 adrenoceptor antagonists.[5] The postjunctional enhancement of the increase in perfusion pressure was likewise not blocked by an α_2 adrenoceptor antagonist but was reduced by the α_1 adrenoceptor antagonist, prazosin, which reduced the initial increase in perfusion pressure produced by the released NE.

Pre- and Postjunctional Effects in the Spontaneously Hypertensive Rat (SHR)

The pre- and postjunctional effects of NPY in the perfused mesenteric artery obtained from 8–10-week-old SHR and Wistar-Kyoto (WKY) controls were examined. It was observed that the prejunctional inhibitory effect was attenuated while the postjunctional potentiation of the response to both nerve stimulation and exogenously administered NE was enhanced.[13,14]

Pre- and Postjunctional Effects of C-Terminal Fragments

The effect of NPY, PYY as well as various C-terminal NPY fragments on the periarterial nerve stimulation induced release of NE is summarized in FIGURE 4. With the exception of NPY 17–36, all of the peptides produced a concentration-dependent decrease in the evoked release of NE. NPY 17–36 produced a biphasic response, increasing release at low and inhibiting it at high concentrations of peptide. The effect of the peptides on the postjunctional response, namely the increase in perfusion pressure, is depicted in FIGURE 5. All of the C-terminal fragments resulted in an attenuation of the increase in perfusion pressure while NPY and PYY produced biphasic effects; slight reduction at low concentrations but marked enhancement at high concentrations. FIGURE 6 depicts a representative record of the perfusion pressure response to nerve stimulation in the absence or presence of varying concentrations of NPY 14–36.

A comparison of the maximal pre- and postjunctional responses exerted by NPY, PYY and the C-terminal fragments is summarized in FIGURE 7. At a concentration of 10^{-7} M

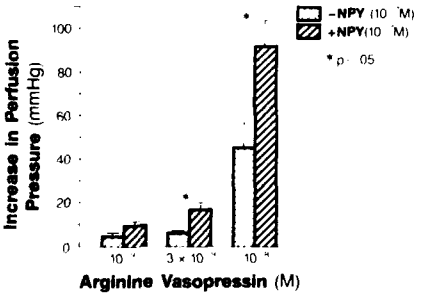

FIGURE 3. The increase in perfusion pressure to arginine vasopressin in the absence or presence of NPY (10^{-7} M). Data are plotted as the increase in perfusion pressure (mmHg) verses increasing concentrations of vasopressin. Each bar is the mean ± SEM of 6–8 observations. NPY produced a potentiation of the increase in perfusion pressure to vasopressin.

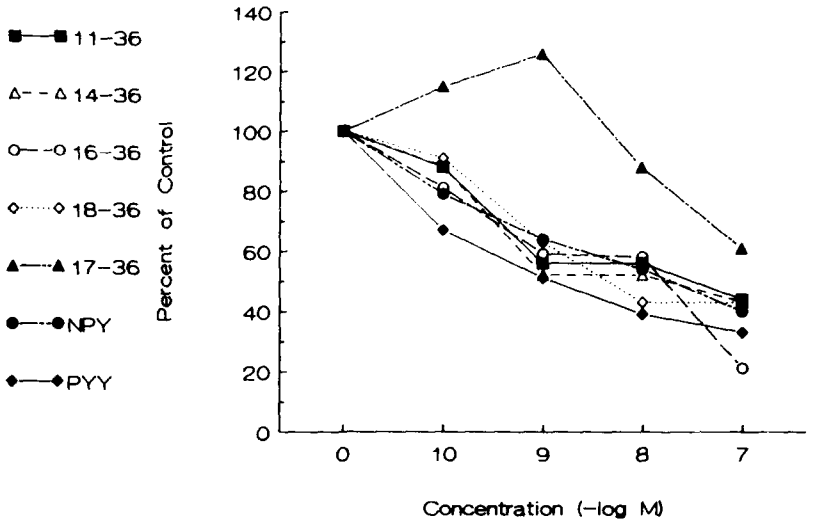

FIGURE 4. The effect of NPY, PYY and NPY C-terminal fragments on the periarterial nerve stimulation-induced release of NE. Data are plotted as percent (%) of control release verses concentration of peptide in M. Each point is the mean of 6–8 observation. NPY, PYY and the various fragments produced a concentration-dependent decrease in the evoked release of NE.

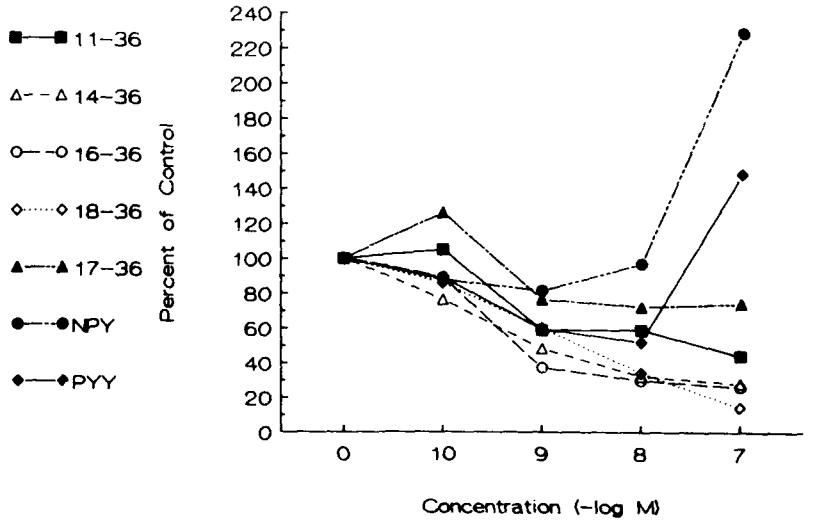

FIGURE 5. The effect of NPY, PYY and NPY C-terminal fragments on the periarterial nerve stimulation-induced increase in perfusion pressure. Data are plotted as percent (%) of control verses concentration of peptide in M. Each point is the mean of 6–8 observations. NPY and PYY produced are enhancement of the increase in perfusion pressure while the fragments produced are attenuation of the response.

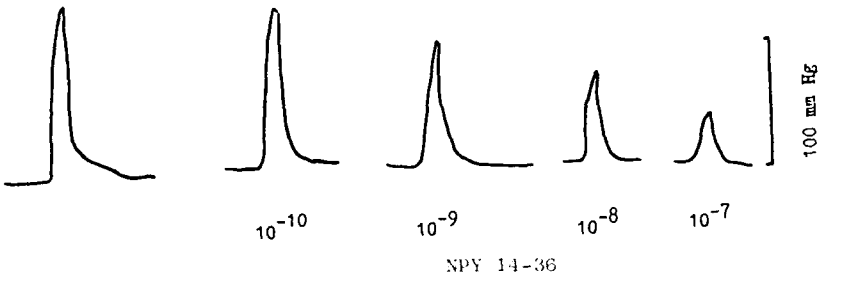

FIGURE 6. Representative tracing of the increase in perfusion pressure due to periarterial nerve stimulation in the absence (*first tracing*) or increasing concentrations of NPY 14–36. NPY 14–36 produced a concentration-dependent reduction in the perfusion pressure response.

all of the fragments produced a parallel decrease in NE release as well as perfusion pressure. In contrast, NPY and PYY produced inhibition of NE release but potentiated the increase in perfusion pressure.

An attempt was made to see if any of the C-terminal fragments could be acting as partial agonists and therefore used as antagonists to NPY in further functional studies. FIGURES 8 and 9 depict the prejunctional and postjunctional effects of NPY in the absence or presence of C-terminal fragments. Both the prejunctional inhibitory effect in NE

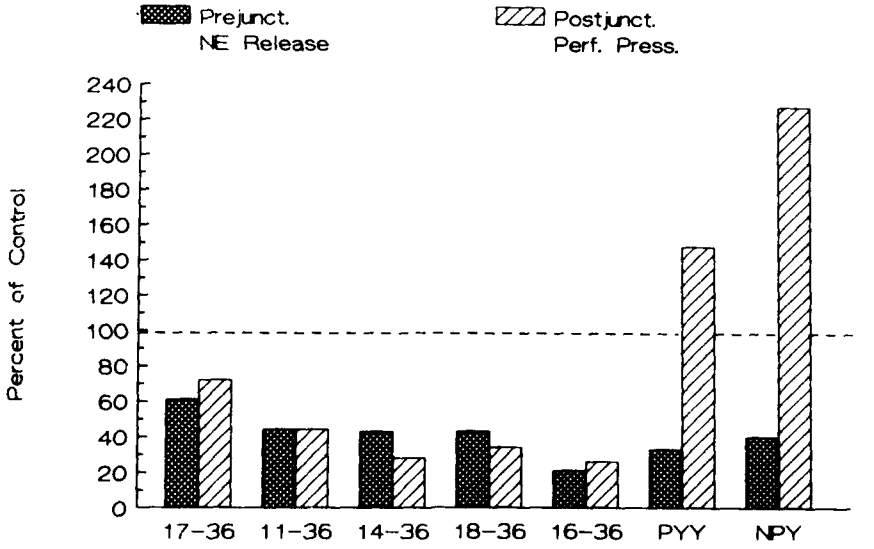

FIGURE 7. The effect of NPY, PYY and NPY fragments at a concentration of 10^{-7} M on the periarterial nerve stimulation-induced release of NE (prejunctional) or increase in perfusion pressure (postjunctional). Data are plotted as percent (%) of the control response taken as 100. The fragments produced a parallel decrease in release and perfusion pressure while NPY and PYY produced an inhibition of release but a potentiation of the increase in perfusion pressure

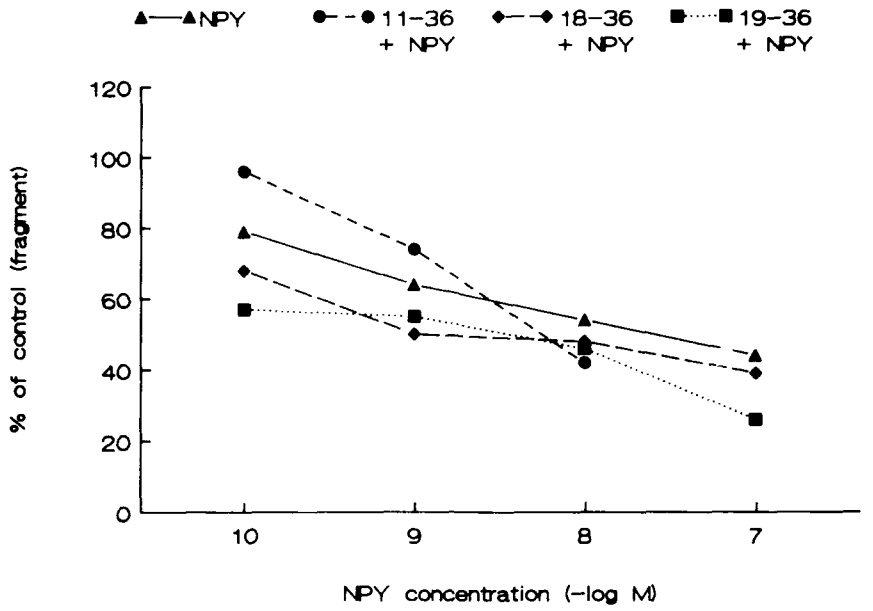

FIGURE 8. The effect of NPY alone or NPY plus various C-terminal fragments on the periarterial nerve stimulation induced release of NE. Data plotted as a percent (%) of the response to the fragments alone. The combination of NPY plus fragments resulted in a similar inhibition of release as with NPY alone or fragment alone.

release (FIG. 8) as well as potentiation of the postjunctional response (FIGS. 9 and 10) produced by NPY were similar in the absence or presence of the C-terminal fragments.

C-Terminal Fragments in the Spinal Cord and Hypothalamic Slices

The intrathecal administration of NPY into the thoracic spinal cord produces a depressor effect as well as hemodynamic changes due to a decrease in sympathetic nerve activity.[11] Similar effects were observed following the intrathecal administration of C-terminal fragments although the fragments were less potent (FIG. 11).

The potassium-induced release of NE from slices of anterior and posterior hypothalamus obtained from various strains of rat was reduced by NPY (FIG. 12). This inhibitory effect was also observed when various C-terminal fragments such as 16–36 were exam-

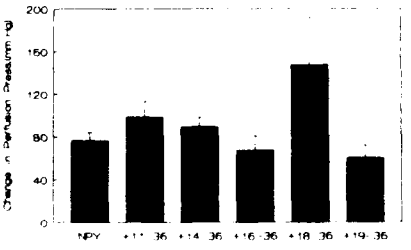

FIGURE 9. The effect of NPY alone or NPY plus various C-terminal fragments on the periarterial nerve stimulation-induced increase in perfusion pressure. Data are plotted as the increase in perfusion pressure (mmHg) + SEM. The *first bar* represents NPY alone (10^{-7} M). The normal potentiation of the increase in perfusion pressure produced by NPY was not altered when administered along with a C-terminal fragment.

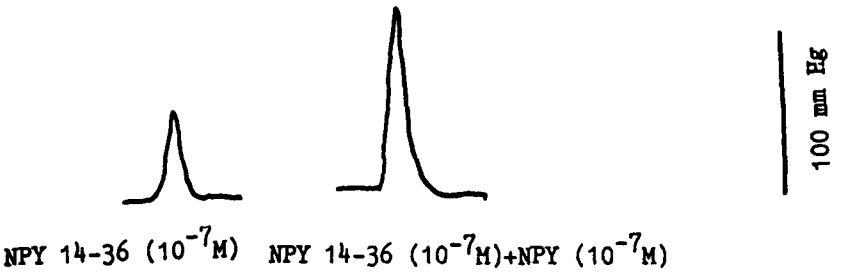

FIGURE 10. Representative tracings of the increase in perfusion pressure to periarterial nerve stimulation in the presence of NPY 14–36 or NPY 14–36 plus NPY. The typical potentiation of the response produced by NPY was not altered when NPY was administered together in the NPY 14–36.

ined (FIG. 13). There was no evidence obtained that the C-terminal fragments could reduce the inhibitory effect of NPY on the potassium-evoked release of NE (FIG. 14).

DISCUSSION

These studies show that NPY produces marked effects at the vascular neuroeffector junction. Using the perfused mesenteric arterial bed as a model of the noradrenergic neuroeffector junction, NPY exerted both prejunctional and postjunctional effects. Prejunctionally, NPY decreased the periarterial nerve stimulation-induced release of NE while postjunctionally it potentiated the nerve stimulation-induced increase in perfusion pressure. Moreover, NPY potentiated the increase in perfusion pressure produced by a variety of vasoactive agents including angiotensin, vasopressin, as well as α-adrenoceptor agonists like phenylephrine or norepinephrine. These results suggest that NPY is acting at the level of transduction mechanisms common to this diverse group of vasoactive substances. Our results also suggest that the postjunctional effect of NPY is independent of endothelial cells.

There is growing evidence for the existence of multiple subtypes of NPY receptors.[13–15] Studies by Wahstedt and colleagues suggest that the postjunctional response requires the entire 1–36 peptide and have called this receptor subtype Y_1.[15–17] In contrast

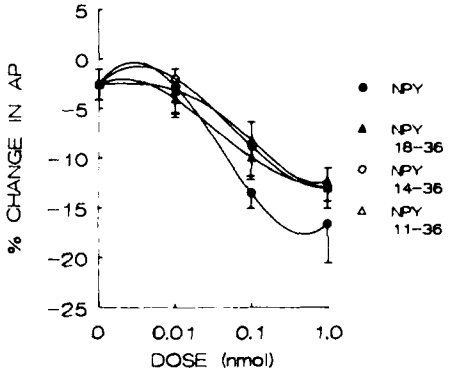

FIGURE 11. The effect of NPY or NPY fragments or the percent (%) change in arterial blood pressure of the anesthetized rat following intrathecal administrative. Data are plotted as the percent (%) change in arterial pressure verses dose of NPY or NPY fragment. NPY and the various fragments all produced a decrease in arterial pressure.

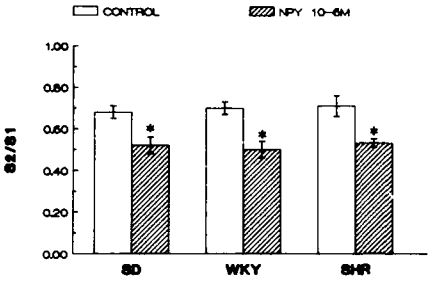

FIGURE 12. The potassium (56 mM) induced release of ^{3}H-NE from slices of the anterior hypothalamus of Sprague Dawley (SD), Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats in the absence (control) or pressure of NPY (10^{-6} M). Each bar is the mean ± SEM of 6–8 observations. Data are plotted at the S_2 (pressure of NPY) /S_1 (absence of NPY) ratio. NPY produced a significant decrease in the potassium-induced release of ^{3}H-NE.

the prejunctional response could be produced by shorter C-terminal fragments (*e.g.*, PPY 13–36 or NPY 13–36) and has been designated the Y_2 receptor.[15-17] Ligand binding studies support the concept of at least two types of NPY receptors that can also be distinguished on the basis of the entire NPY sequence or shorter NPY fragments.[18-19] Y_2 receptors bind long C-terminal fragments while Y_1 receptors bind the entire amino acid sequence. In addition, there are also NPY analogs that are specific for the Y_1 receptor.[20] Further support for the Y_1 and Y_2 concept is data suggesting they are coupled to different transduction mechanisms.[21] For instance, in the human neuroblastoma cell line SK-N-MC NPY and the specific Y_1 receptor legend (Leu^{31}, Pro^{34})-NPY caused a rapid and transient increase in the concentration of free calcium in the cytoplasm as well as inhibited the forskolin-stimulated cAMP production. Y_2 specific ligands had no effect on either intracellular second messenger[19] Our results utilizing NPY, PYY, and a series of NPY C-terminal fragments are consistent with the Y_1–Y_2 concept. The strength of our study is that pre- and postjunctional responses were examined simultaneously in a model noradrenergic vascular neuroeffector junction. Our results are consistent with the postjunctional response (Y_1) requiring both the N- and C-terminal while the prejunctional response requires only the C-terminal end of the peptide. Although it may be premature or even simplistic to think that there is a pre-postjunctional distribution of Y_1 and Y_2 receptors, our results do suggest that the predominant postjunctional receptor is the Y_1 subtype while the predominant form of the prejunctional receptors is of the Y_2 subtype. Because of the known colocalization of NPY and NE in noradrenergic neurons innervating the mesenteric arterial bed, our results reinforce the idea that NPY acts as an important cotransmitter/modulator at this vascular neuroeffector junction.

The biphasic effect of NPY 17–36 deserves further comment. It is of interest that NPY and the COOH terminal fragments 13–36, 16–36, 19–36, 20–36 and 25–36 all inhibited both basal and isoproterenol-stimulated adenylate cyclase activity in rat cardiac ventricular membranes[22] In contrast NPY 17–36 stimulated basal and enhanced the isoproterenol-stimulated adenylate cyclase activity. In the present studies NPY 17–36 stimulated

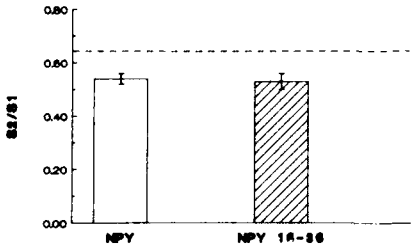

FIGURE 13. The effect of NPY (10^{-6} M) or NPY 16–36 (10^{-6} M) or the potassium-induced release of endogenous NE from hypothalamic slices of Sprague Dawley rats. Data are plotted as an S_2/S_1 ratio similar to FIGURE 11. The *dotted line* represents release of NE to potassium alone while the *bars* depict release in the pressure of NPY or NPY 16–36. All the fragments studied produced a significant increase in the potassium-induced release of NE similar to NPY itself.

release of NE except at high concentrations while NPY, PYY and all the other fragments produced only inhibition of NE release. Since there are data suggesting that increases in cAMP enhance transmitter release, it is suggestive of cAMP being involved in the prejunctional actions of NPY. Further studies are, of course, needed to further probe this idea.

Our results also implicate NPY in the pathophysiology of hypertension development and/or maintenance in the SHR. At the vascular neuroeffector junction of the mesenteric arterial bed, the postjunctional potentiation of contraction to vasoactive agents and nerve stimulation by NPY was enhanced. In contrast, the prejunctional effect of inhibition of NE release was attenuated. This combination of responses would result in a greater increase in blood pressure. The mechanism of these changes is not understood. However, since there is evidence for subtypes of NPY receptors, it is possible that there might be differential up and down regulation of Y_1 and Y_2 receptors. It is also possible that there may be differential effects on the intracellular transduction mechanisms. To date NPY has been shown to inhibit the stimulation of cAMP induced by various agents,[23,24] to increase phosphoinositide turnover,[25,26] to produce an increase in intracellular calcium[26,27] or to inhibit intracellular Ca^{2+} transient and depolarization induced transmembrane Ca^{2+} currents.[28–31] Alterations in one or more of these transduction mechanisms may occur in blood vessels or neuronal tissue of the SHR.

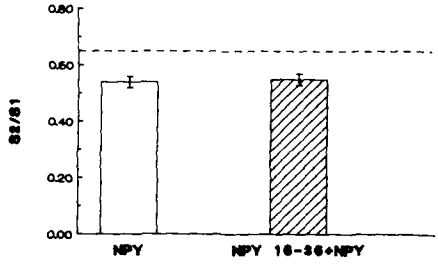

FIGURE 14. The effect of NPY alone (10^{-6} M) a NPY (10^{-6} M) plus NPY 16–36 (10^{-6} M) on the potassium-induced release of endogenous NE from hypothalamic slices. Data are plotted in a similar fashion to FIGURE 12. The pressure of the NPY fragments did not alter the inhibition of release produced by NPY alone.

The results of studies carried out with C-terminal fragments in the spinal cord and hypothalamus suggest that the depressor effect following intrathecal administration and the inhibition of NE release from hypothalamic slices may be due to activation of Y_2 receptors.

CONCLUSION

In conclusion, our studies have demonstrated that NPY produces prejunctional inhibitory and postjunctional excitatory effects at the noradrenergic neuroeffector junction of the mesenteric arterial bed. The postjunctional effect is nonspecific in that NPY potentiated the increase in perfusion pressure to a number of diverse substances suggesting an effect on a common intracellular transduction mechanism. The postjunctional effect is independent of endothelial cells and appears due to activation of Y_2 receptors while the prejunctional effect is due to activating the Y_1 receptor subtype. The prejunctional effect of NPY was attenuated while the postjunctional effect was enhanced in beds obtained from the SHR. Our results therefore implicate an alteration in the NPY system in the development or maintenance of hypertension in the SHR.

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Importance of Neuropeptide Y in the Regulation of Kidney Function[a]

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-aminoacid peptide structurally related to the pancreatic peptide family. It has previously been shown to exist in sympathetic nerves and in central nervous tissue.[1-4] Within the sympathetic nerve terminals NPY is stored in large dense-cored vesicles.[5,6] Not only is NPY co-localized with norepinephrine, it is also co-released during nerve stimulation[7,8] and interacts with norepinephrine on the pre- and postsynaptic level.[1,9,10]

The physiological effects of NPY are similar to noradrenergic stimulation, but systemic administration of NPY will produce hypertension, which is not blocked by adrenergic antagonists.[11] NPY does not only increase vascular resistance by this adrenoceptor-independent mechanism, it also potentiates vasoconstrictor responses to norepinephrine and sympathetic nerve stimulation.[12] The kidney possesses a particularly high concentration of NPY, as do the coronary and mesenteric arteries.[1,13] Furthermore, the kidney reveals a particularly high density of high-affinity binding sites for this peptide.[14] Hence, NPY may play an important role in modulating a variety of renal processes. In fact, studies in the rat have provided evidence for an influence on renal sodium reabsorption[15] and renin release.[16,17] These observations are strengthened by the fact that NPY has been localized in the sympathetic nerve fibres at the juxtaglomerular apparatus,[18] the site of renin secreting cells.

In addition to these direct effects, NPY might also modulate renal function via its interaction with the adrenergic transmitters. It is well documented, that sympathetic nerves have a profound impact on sodium excretion.[19,20] Pressure-dependent renin release is also modulated by sympathetic activation,[20,21] and even the range of renal autoregulation is dependent on the prevailing sympathetic tone to the kidney.[22]

Characterization of Renal NPY Receptors

Binding sites for NPY have been reported in a variety of tissues including the brain,[23] spleen,[24] and aorta.[25] A vast amount of high-affinity binding sites has also been found in the rabbit kidney.[14] Studies employing autoradiographic techniques in the kidney have demonstrated that NPY binding is not restricted to the vascular tissue. The proximal

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convoluted tubules reveal binding as well.[26] This seems to fit nicely to the proposed effects of NPY on sodium excretion as will be referred to below.

A recent study[27] describes a technique for solubilizing NPY receptors from the rabbit kidney using the zwitterionic detergent 3-[(3-cholamidopropyl)dimetylammonio]-1-propanesulfonic acid (CHAPS). The receptor characteristics were not altered by this procedure as indicated by saturation studies revealing very similar k_D values (0.10 ± 0.02 nM for membranes vs. 0.12 ± 0.03 nM for soluble extract). A Scatchard analysis of the equilibrium binding data demonstrated a single class of binding sites (FIG. 1). A 100-kDa polypeptide represents the covalently labeled NPY receptor. This was shown by affinity labelling experiments using two different categories of cross-linking agents. The specificity of the labelling was apparent by the ability of unlabeled NPY to displace the binding.

The nonhydrolizable GTP analog (GTPγS) specifically inhibited NPY binding. Within 5 min of incubation the specific ^{125}I-NPY binding decreased to 15% of control (FIG. 2) for the solubilized fraction. This suggests that these NPY receptors are functionally coupled to GTP-binding regulatory proteins. This agrees with some indirect evidence obtained in other tissues, that at least one type of NPY receptors belongs to the class of G-protein coupled receptors.[24,28,29]

Considerable evidence derived by investigations on the rat brain and cell lines of neuronal origin speaks in favor of two NPY receptor subtypes.[30,31] Until further experiments utilizing these ligands have been performed in the kidney, the question about the presence of both subtypes in the kidney remains unanswered.

An interesting observation of rabbit kidney NPY receptors, however, is the competitive action between NPY and the humoral peptide YY.[14,27] Perhaps circulating peptide YY also acts as a physiological ligand at the kidney NPY receptor.

NPY Localization and Distribution throughout the Kidney

With the use of several region-specific antisera and the peroxidase-antiperoxidase technique, Reinecke and Forssmann[32] found a wide distribution of NPY in all segments of the renal vasculature. In comparison to the large number of polypeptides investigated in renal nerves, NPY was the most prominent. The nerve fibres containing NPY were located homogeneously around large renal arteries continuing into a dense plexus of the arcuate and interlobular arteries. An equivalent density of NPY nerves occurred around the afferent and efferent arterioles.

The juxtaglomerular apparatus is a neuroendocrine unit located at the vascular pole of the glomerulus and is known to have a rich supply of monoaminergic nerve fibres. In this dense plexus of fibres of several species including man, NPY has also been located by means of immunocytochemistry.[18] This finding is of particular importance since the juxtaglomerular apparatus is a crucial component for renin secretion. In the mentioned study, NPY-LI immunoreactivity was also markedly depleted after chemical sympathectomy by 6-hydroxydopamine.

In yet another study,[26] *in vitro* labelling of NPY binding sites with ^{125}I-NPY was performed in rabbit kidney sections. Autoradiographic and histochemical techniques identified binding sites not only in the vascular smooth muscle, but in the proximal tubules as well. Intriguingly, however, no binding was found in the rat, guinea pig and human kidney. Hence, for the time being, an unequivocal interpretation of these partly conflicting results is not possible, but the preferential localization of NPY to certain segments of the kidney may provide insight into its physiological and pathophysiological importance.

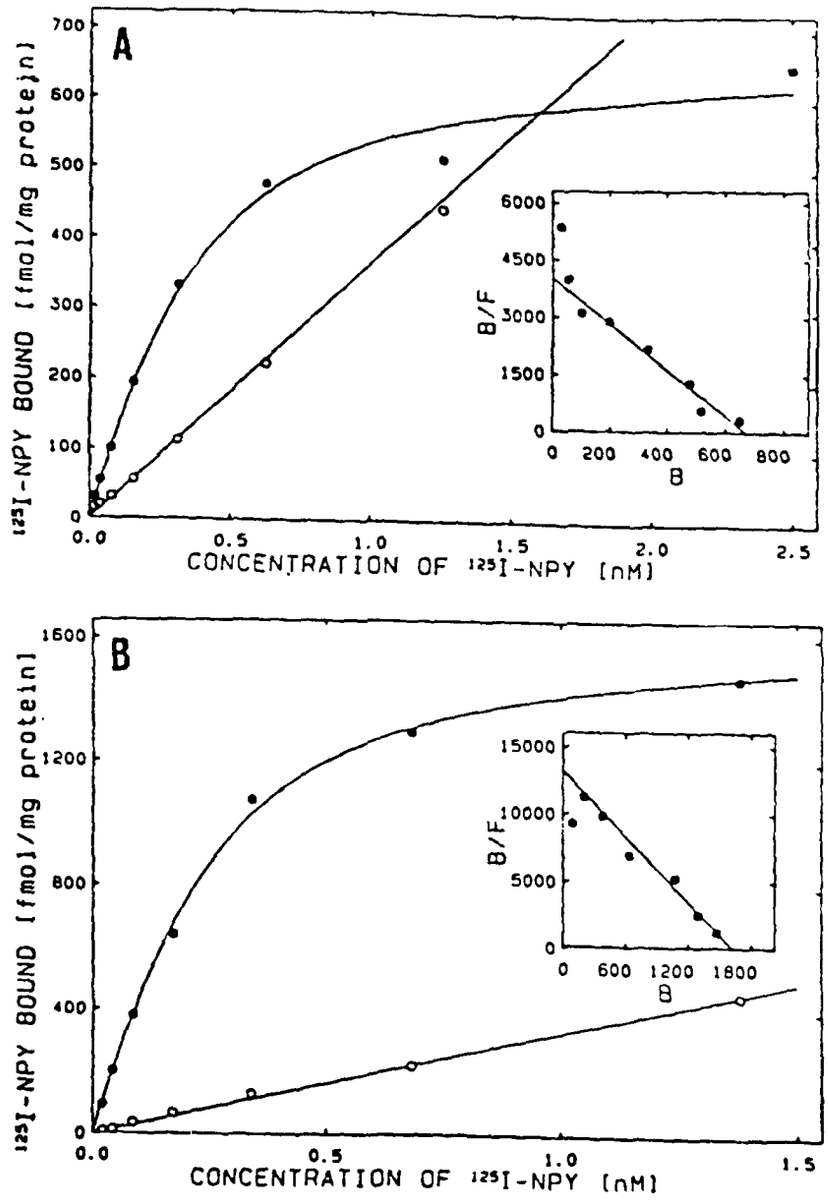

FIGURE 1. Saturation curves and Scatchard analysis of NPY binding. **(A)** Membrane preparation; **(B)** CHAPS solubilized fractions. *Open circles* refer to unspecific binding; *filled circles* represent the specific binding estimate (unspecific-total ligand bound). The insets show the corresponding Scatchard plots (B: specifically bound NPY, F: free NPY in nM).

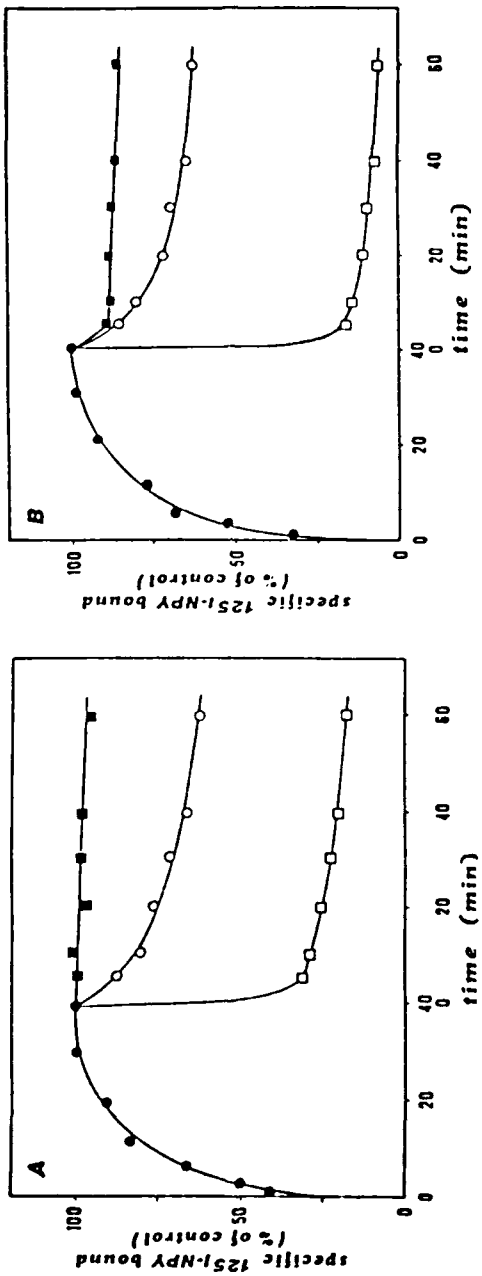

FIGURE 2. Association kinetics (*filled circles*) of ^{125}I-NPY (0.4 nM) to rabbit kidney membrane (**A**) and to CHAPS solubilized extracts (**B**). Dissociation by unlabeled NPY (final 1 μM) is demonstrated by the *open circles*. The *open squares* refer to parallel experiments in which unlabeled NPY was added in combination with GTPγS (final 0.1 mM). As a control, ATPγS was added instead of GTPγS at the same concentration (*filled squares*).

NPY and Renin Release

Juxtaglomerular renin secretion is modulated by numerous local and systemic influences. It is remarkable that all effectors of renin secretion have one feature in common: vasoconstricting agents generally inhibit, and vasodilators augment renin secretion.[33] There can be no doubt that NPY is a potent vasoconstrictor; thus, according to this general rule, one should expect NPY to act in an inhibitory fashion on renin secretion. Indeed, an inhibition of renin secretion by NPY was described by Hackenthal and colleagues[16] in the isolated rat kidney. Since perfusion pressure is known to be one of the most important factors influencing renin release,[34] the kidney was perfused at a constant pressure (105 mmHg) by means of a servocontrol mechanism. In this preparation NPY produced a dose dependent inhibition of renin release, which corresponded to the degree of vasoconstriction. However, the baseline renin secretion increased steadily throughout the experimental protocol. This seemingly paradox behavior is not necessarily due to a specific NPY effect, since it was also demonstrated by other vasoconstricting agents, such as angiotensin II.

To exclude a simple flow-dependent or "washout" effect on renin release, these authors repeated their study under constant flow conditions; furthermore, tissue samples were studied taken from the hydronephrotic rat kidney. All approaches yielded the same results: NPY had a strong inhibitory effect on renin release. Pertussis toxin clearly attenuated the response to NPY; thus an adenylate cyclase coupled mechanism was suggested.

The inhibition of renin release by NPY is supported by a recent study in anesthetized cats.[35] Pressor and nonpressor intravenous (iv) NPY doses decreased plasma renin activity. Intriguingly, the same "rebound effect" of renin release as seen by Hackenthal *et al.*[16] was observed. The baseline level of plasma renin activity increased substantially as soon as the infusion ended.

The harmonious results of these two studies is challenged by the work of Echtenkamp and Dandridge.[36] They studied the effects of NPY administered either intravenously or directly into the renal artery of anesthetized primates. Increasing iv NPY doses from 20 to 1000 ng/kg/min, as well as intrarenal NPY administration between 20–400 ng/kg/min NPY did not have any significant effects on plasma renin activity or renin release, in spite of renal vasoconstriction at the higher NPY levels. This is substantiated by a recent study showing that iv infusions of NPY in the rat did not influence plasma renin activity.[37] A lack of NPY inhibition on renin release has also been found during iv NPY infusions in normal rats when only modest increases in arterial blood pressure occurred.[17] However, in the same study NPY normalized renin release in adrenalectomized rats without concomitant changes in blood pressure. Accordingly, NPY might exert an indirect influence on renin release via its interaction with adrenergic transmitters. α- and β-adrenergic effects on renin release are well documented,[20,21] and NPY inhibits nerve stimulation evoked norepinephrine release in several tissues including the kidney.[38,39] To test this hypothesis, an experimental protocol was developed in the conscious dog in which a reflex sympathetic stimulus could be elicited and renal perfusion pressure was controlled (FIG. 3). Dose response curves were obtained by infusing NPY into the renal artery and measuring the reductions in renal blood flow (RBF). A dose was chosen for the experiments which was just below the dose necessary to reduce RBF (1 μg/min). This infusion increased renal venous NPY concentrations from roughly 40 to 300 pg/ml. The preliminary data depicted in FIGURE 3 show that renin release was not decreased by this procedure, if at all; there might even be a slight increase during NPY infusions. The next step was to determine whether these effects were the same at a reduced level of renal artery pressure. This is important since there is a threshold pressure for renin release (roughly 95 mmHg).[34] Above this limit the effects on renin release may be negligible or at least difficult to detect. Renal artery pressure was servocontrolled by an electropneumatic control system[34] at 80 ± 0.5 mmHg. The surprising finding was that during the low

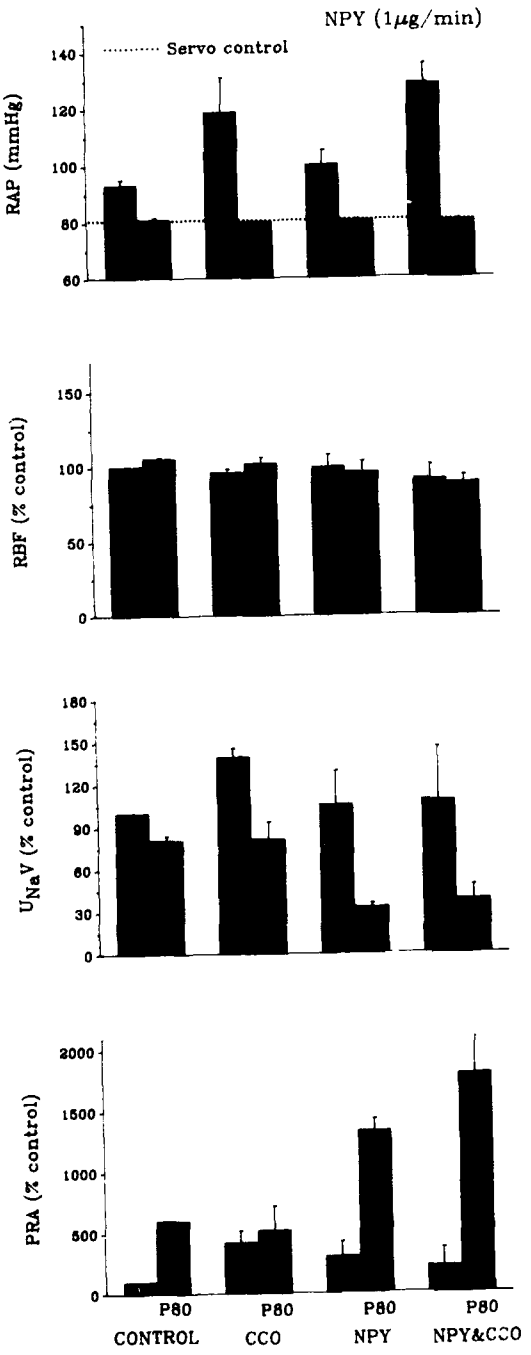

FIGURE 3. Effects of intrarenal NPY-infusions (1 μg/min, *right*) compared to no infusions *(left)*. Common carotid occlusion (CCO) was used as a sympathetic stimulus. Renal artery pressure could be servocontroled at 80 mmHg (P80). The data are means from 6 experiments in 3 dogs.

dose NPY infusion renin release increased much more than under control conditions. It should be mentioned, that 80 mmHg is well above the lower limit for RBF autoregulation;[22] hence, the NPY infusion did not alter total renal vascular resistance (FIG. 3).

In order to quantify the interaction between sympathetic activation and simultaneous NPY infusion, we repeated this experimental protocol during common carotid occlusion (CCO). The results indicate, that there is no apparent difference between the protocols with or without the reflex sympathetic activation. This, on the other hand, agrees with findings from Hackenthal and colleagues[16] made in the isolated rat kidney. They found that the α_1-adrenoceptor agonist prazosin (2 μM) only slightly modified the renin release response due to NPY.

Taken together, there is disagreement towards several important features concerning the effects of NPY on renin release. It has, however, been shown, that there is a rebound phenomenon of renin release after NPY infusions. This phenomenon cannot be explained at present, but it does not seem to be confined to NPY, since similar findings were made for other vasoconstrictors. Furthermore, it does not seem likely that NPY and adrenergic transmitters specifically interact in the regulation of renin release. Nevertheless, at this point, it is not possible to clarify the crucial question if NPY mediates its effects on renin release via a direct action on the juxtaglomerular apparatus, or if these effects may be secondary to its vasoconstrictor actions.

Renal Vascular Resistance

More insight might be obtained by the data concerning renal vascular resistance, and especially by the electrophysiological recordings from epithelioid cells.

In a number of *in vitro* and *in vivo* preparations, exogenous NPY exerts a direct postsynaptic vasoconstriction and potentiates the vasoconstrictor action of other agents.[15,40,41] Although, NPY induced vasoconstriction has been described in most vascular beds, the vasoconstricting potency varies considerably between different organs. Pernow and Lundberg[38] found a slight reduction in RBF by exogenous NPY in very small doses, which resulted to an arterial plasma NPY-LI concentration of 164 pmol/l. This demonstrates a remarkable sensitivity of the renal vasculature to NPY since other beds respond with vasoconstriction at nanomolar concentrations.[39] In the same study, the effects of renal nerve stimulation on RBF were investigated before and after adrenergic blockade. During high frequency stimulation (10 Hz) there were still mentionable reductions of RBF inspite adrenergic blockade. It is likely, that this effect is due to NPY release, as indicated by a high correlation between NPY-LI overflow and the % reduction of RBF.

By injecting considerable amounts of NPY (10 μg/kg as a bolus) in the conscious rabbit, Minson and colleagues[40] detected a marked increase in renal vascular resistance, a moderate rise in mesenteric vascular resistance, and a transient fall in hindlimb vascular resistance. Taken together, there is data indicating (i) a high renal vascular sensitivity to NPY, (ii) a reduction of RBF during high frequency renal nerve stimulation in the presence of adrenergic blockers, and (iii) differential effects of high dose NPY administration on regional vascular resistance. Thus, the following hypothesis can be made: In situations with a high sympathetic outflow, NPY may redistribute blood flow in favor of the skeletal muscle. This would suggest a participation of NPY in the "fight or flight" reflex.

Intriguingly, preliminary data suggests, that the effects of NPY are also not evenly distributed throughout the kidney. Bührle and colleagues[42] investigated the membrane potential of renin containing epithelioid and vascular smooth muscle cells of the afferent arteriole in the hydronephrotic mouse kidney. Several agents, such as angiotensin II, and

α_1-adrenoceptor agonists depolarized both types of cells, but NPY was without effect. This was at roughly 25 μm distance from the point of entry into the glomerulus. Further upstream (> 100 μm from the point of entry), there is a depolarization, as indicated by FIGURE 4. This is in agreement to *in vivo* microscopy of the hydronephrotic kidney. In this preparation, NPY mainly constricted larger preglomerular vessels (Steinhausen, personal communication).

A predominant constriction of larger renal vessels by NPY may reconcile the conflicting data concerning renin release. If NPY were to exert a preferential vasoconstriction on the larger vessels upstream from the glomerulus, then the autoregulating kidney would respond with a subsequent dilatation in the smaller vessels downstream. Vasodilation of these vessels will then augment renin release. This mechanism is most apparent at lower perfusion pressures, due to a reduced vasodilatory reserve in the renal vascular bed (autoregulation).

Sodium Excretion

Not much is known about the NPY actions on sodium excretion. Allen and colleagues[15] used an isolated rat kidney preparation to investigate this issue. A rather unexpected finding was the natriuretic effect of NPY despite renal vasoconstriction. The

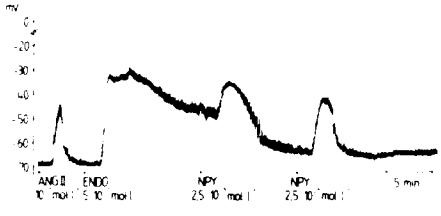

FIGURE 4. Effects of NPY upon the membrane potential of a smooth muscle cell more than 100 μm from the entry point into the glomerulus. As a comparison: angiotensin II (ANG II) and endothelin (ENDO).

perfusion flow as well as the glomerular filtration rate did not overly increase during 200 pmol NPY, and sodium excretion after NPY appeared disproportionate to the pressure effect. Therefore, the authors suggested a natriuretic action, which is more or less independent of hemodynamic alterations. However, the perfusion pressure did increase by about 15 mmHg even at these lower NPY levels. In the conscious dog, such an increase in pressure would be more than enough to explain the natriuresis in this study.[19]

Echtenkamp and Dandridge[36] do not confirm a natriuretic effect of NPY. The *in situ* kidney of uninephrectomized primates revealed a dose dependent antinatriuresis. The sodium retention commenced at doses of NPY that decreased RBF and glomerular filtration rate. Hence, it was impossible to demonstrate a direct tubular effect of NPY.

This is in accord with findings depicted in FIGURE 3. No effects of NPY were found at or above control pressure in the conscious dog. Interestingly, however, the antinatriuresis caused by reducing renal artery pressure to 80 mmHg was augmented. This again may be sufficiently explained by hemodynamic alterations: NPY vasoconstriction may impair the range of renal autoregulation. An impairment of renal autoregulation has recently been shown by even modest sympathetic reflex activations.[22]

Thus, it has not been possible to detect a consistent NPY effect on sodium excretion without the obscuring changes in renal hemodynamics. The latter seem to have a stronger impact on sodium excretion than any measurable NPY effect. However, sodium excretion

of the rabbit may be different from these other species, since NPY binding has been detected in the rabbit proximal convoluted tubules.

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Neuropeptide Y and Sympathetic Neurotransmission[a]

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INTRODUCTION

Neuropeptide Y (NPY) is a peptide with 36-amino acid residues which was originally isolated from the porcine brain[1] and probably represents the earlier described neuronal pancreatic polypeptide-like immunoreactivity.[2] A major portion of the NPY-immunoreactive nerves in peripheral organs represents classical sympathetic fibres where NPY co-exists with noradrenaline (NA).[3,4] NPY is thus present in perivascular sympathetic fibres as well as in the noradrenergic nerves to the muscle of the heart, spleen, and vas deferens.

Vasoconstrictor Actions of NPY

Exogenous NPY causes potent, long-lasting reduction in local blood flow in many organs of experimental animals[5,6] (FIG. 1) as well as in the human forearm.[7] *In vitro*, NPY contracts small isolated blood vessels such as cerebral,[8] splenic,[9] renal and skeletal muscle arteries[10] with threshold effects in the nM concentration range. The NPY effect is mediated via activation of specific nonadrenergic receptor mechanisms and the vasoconstriction is characterized by a slow onset and a long duration. The vasoconstrictor effect of NPY is independent of the vascular endothelium suggesting an action directly on vascular smooth muscle cells.[9] In the human forearm local i.a. infusion of NPY causes both reduction in blood flow and increase in venous tone suggesting constriction of both resistance and capacitance vessels[7] in analogy with data from pig nasal mucosa.[11]

NPY Receptors and Intracellular Messengers

Calcium antagonists like nifedipine inhibit the vasoconstrictor response to NPY on renal and skeletal muscle arteries but not on mesenteric veins.[10] The NPY effect was largely uninfluenced by changes in extracellular Ca^{2+} concentrations suggesting that NPY rather acts via changes in intracellular Ca^{2+} (see REF. 10). NPY does not stimulate inositol phosphate turnover in blood vessels per se[12] while the forskolin-stimulated cyclic AMP formation in vascular smooth muscle[12] and heart muscle[13] is inhibited. High affinity-binding sites for NPY with receptor characteristics have been demonstrated in both

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blood vessels and the capsule of the pig spleen.[12,14,15] A large amidated C-terminal portion of NPY is a prerequisite for receptor binding, inhibition of forskolin-stimulated cyclic AMP formation and vasoconstrictor effects.[12,14] The structurally related peptide YY (1–36) binds to NPY receptors with similar or larger affinity than NPY and also evokes potent vasoconstriction.[5,12,14,15]

Supersensitivity to the vasoconstrictor effects of NPY after sympathetic denervation has been observed in the rat tail artery *in vitro*[16] and in the pig nasal mucosa *in vivo*[17] but not in the pig spleen.[14]

NPY and "Nonadrenergic" Vasoconstriction

In the submandibular salivary gland of the cat it was demonstrated[5] that NPY mimicked the slowly developing and long-lasting decrease in blood flow evoked by sympathetic nerve stimulation using a high frequency in the presence of α- and β-adrenoceptor

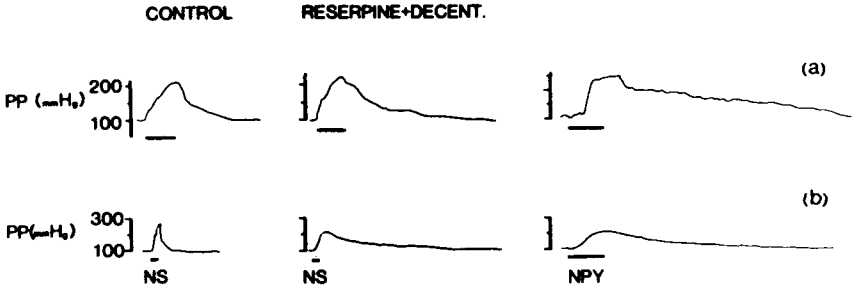

FIGURE 1. Effects of electrical sympathetic nerve stimulation (NS, bar) and local i.a. infusion of NPY (2 nmol/min) on vascular tone as revealed by perfusion pressure (PP, mmHg) in **(a)** cat spleen (10 Hz for 2 min) and **(b)** dog gracilis muscle (6.9 Hz 30 sec). The nerve stimulation evoked vasoconstriction (increase in perfusion pressure) in controls is markedly prolonged in the animals where reserpine pretreatment (1 mg/kg i.v.) was combined with preganglionic decentralization to maintain tissue NPY levels and to deplete NA by 99%. This long-lasting nerve response is mimicked by NPY infusion. For details see REFERENCES 23,24.

antagonists. Treatment with reserpine in order to deplete (by 99%) the tissue stores of NA may represent an alternative approach to study nonadrenergic transmission since it cannot be excluded that the doses of adrenoceptor antagonists used are not high enough to eliminate all the effects of the released NA. It is well established, however, that only minor vasoconstrictor responses to nerve stimulation in, *e.g.*, the spleen remain in reserpine treatment animals, apparently leaving a minor or no role for nonadrenergic mechanisms.[18] Interestingly, recent studies have shown that reserpine treatment is associated with a reversible depletion not only of NA but also of NPY from sympathetic nerve fibres in a variety of tissues including heart, skeletal muscle and spleen.[19–21]

Several characteristic features separate the effects of reserpine pretreatment on NA storage from that on NPY. Thus, higher doses of reserpine are required to deplete NPY than NA. The depletion of NPY is slower in onset and less pronounced than that of NA in terminal regions.[19,21] Furthermore, the NPY content of sympathetic axons and in terminals of some tissues like the vas deferens is not reduced after reserpine suggesting an action which is not directly related to storage mechanisms.[20,21] Most strikingly, the

reserpine-induced reduction of tissue NPY levels is, in contrast to the effect on NA, entirely dependent on an intact nerve activity since either pretreatment with a nicotinic receptor blocking agent such as chlorisondamine, surgical transection at the pre- or postganglionic levels or clonidine, which reduces sympathetic discharge, impairs the reserpine effect on NPY but not on NA levels.[21-29] The reserpine-induced depletion of NPY is therefore likely to be caused by an increased release of the peptide in excess of synthesis and resupply capacity by axonal transport (see REF. 25). It is known that reserpine pretreatment is associated with a rapid increase in firing rate of postganglionic sympathetic nerves.[26] Treatment with reserpine can therefore be combined with pharmacological agents or surgical procedures in order to prevent the increased neuronal activity to the organ studied which after 24 h results in a situation where NA is depleted by 98–99%, while the tissue content of NPY is preserved. Stimulation of the sympathetic nerves in the spleen with high frequency then evoked a marked long-lasting vasoconstrictor response which was mimicked by the action of exogenous NPY[23] (FIG. 1). This experimental approach has now been tested in a variety of preparations *in vivo* in addition to the cat spleen,[23] *i.e.*, the pig spleen,[28] dog skeletal muscle,[24] pig nasal mucosa[11] and pig kidney[29] with similar results. The high correlation ($r = 0.79–0.91$) between the vasoconstrictor effects and the detectable overflow of NPY into the local venous effluent, the characteristic time course of the response being slowly developing and long-lasting and fatigue of the response upon repeated stimulation in these reserpinized preparations further favour a peptide like NPY as the mediator. Although release of NPY seems to be facilitated by high frequency stimulation, NPY outflow is detected both from spleen[28] and kidney[29] already at stimulation with a low frequency like 0.5 Hz. Furthermore, in reserpinized pigs, nasal vasoconstriction is observed even in response to single impulses[11] suggesting that exocytosis of the NPY content from large dense cored vesicles can occur also under such conditions although to a lesser extent than at high frequency stimulation.

Following reserpine treatment the levels of NPY in sympathetic ganglia are elevated and an increased accumulation of peptide occurs above an axonal ligation suggesting both enhanced synthesis and subsequent anterograde axonal transport.[20] Accordingly, recent data have shown that expression of specific NPY messenger RNA in sympathetic ganglion cells is increased following reserpine administration.[30] The observation that subchronic pretreatment with chlorisondamine reduces the NPY content in sympathetic ganglion cells[22] further supports the concept of a nicotinic receptor-stimulated regulation of NPY synthesis.

Co-Release of NPY and NA upon Sympathetic Activation

Release of NPY, as revealed by either overflow into the local venous effluent from organs of experimental animals[28,29,31,32] or human heart[33] as well as increases in systemic plasma levels in man[34,35,36] occurs upon sympathetic activation. The ratio between the overflow of NPY and that of NA increases with the frequency of stimulation[28] and the correlation between the NPY and NA overflow represents a sigmoid curve,[37] suggesting that NPY release is preferentially facilitated by high stimulation frequencies[28,37] (FIG. 2). The local plasma concentration of endogenous NPY-LI in the pig splenic or renal venous effluent upon high frequency stimulation in reserpinized animals is in the nM range, *i.e.*, similar to where exogenous NPY evokes vasoconstriction.[6,28,29] Since further characterization by high performance liquid chromatography suggests that the NPY-LI detected by the antiserum (N1) used represents NPY (1–36);[28] this suggests that endogenous NPY concentrations which are likely to be much higher close to release sites are more than sufficient to activate receptors on vascular smooth muscle cells and to contribute to the functional response. Upon prolonged stimulation (evoked by either increased sympathetic

discharge as for reserpine or electrical stimulation), NPY release cannot be maintained and tissue content of NPY is then reduced, however.[28]

The mechanisms underlying the frequency-dependent differential secretion of NPY may be related to the partly separate vesicular storage of these two agents in the sympathetic nerve terminals, whereby NPY seems to be exclusively present in the large dense-cored vesicles[28] (FIG. 3). Pretreatment with guanethidine inhibits the stimulation-evoked release of both NPY and NA,[31] while tyramine evokes NA secretion without influencing NPY indicating that separate release can occur.[32] After α_2-adrenoceptor antagonists the NPY and NA overflow is enhanced in parallel, however[25,31] (FIG. 3).

The plasma levels of NPY in healthy human subjects are mainly elevated upon heavy physical exercise (FIG. 3) or other situations with a strong sympathetic activation such as after hypoxia[33] or adrenoceptor blockade.[34–36] Therefore, also in man the secretion of NPY as detected by elevated systemic plasma levels seems to be enhanced at high degrees of sympathetic activation compared to that of NA. Most species including man have

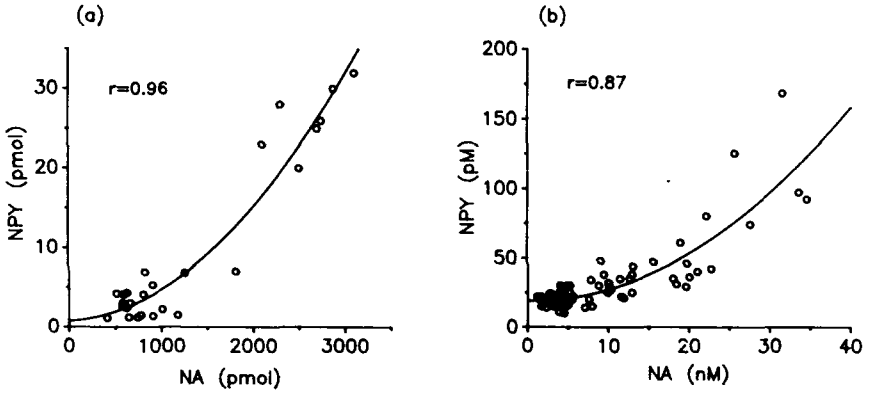

FIGURE 2. Correlation between **(a)** output of NPY (pmol) and NA (pmol) from the blood perfused pig spleen upon sympathetic nerve stimulation with different frequencies (r = 0.96) and **(b)** levels (pM) of NPY and NA in human venous plasma upon graded physical exercise (r = 0.87). Note the similar relative increase in NPY at high NA levels both from pig spleen and humans. For details see REFERENCES 28, 34, 36.

relatively low resting plasma levels of NPY suggesting that the basal rate of NPY release is low, if any.[25] In the rat, however, very high plasma NPY levels are present also during basal conditions which most likely is related to the occurrence of NPY in trombocytes of this species.[38] Therefore, NPY levels in rat blood may not be an appropriate indicator of sympathoadrenal activity[26] but mainly reflect the degree of trombocyte degranulation,[39] especially since plasma NPY in the rat is very sensitive to vinblastine[40] treatment, which markedly reduces trombocyte numbers in peripheral blood.

NPY as Pre- and Postjunctional Modulator of Sympathetic Transmission

NPY or PYY exerts prejunctional actions on release of transmitter from sympathetic nerves as revealed by inhibition of nerve-evoked contractions[3] and stimulation-evoked endogenous NA and NPY output *in vivo*[41,42] or ^{3}H-NA overflow from perivascular nerves

of rat and man *in vitro*[10,43] (FIG. 3). Experimental data studying contractile responses or analysis of transmitter overflow suggest that NPY also inhibits the release of both acetylcholine and NA in the heart.[44-46]

In the mouse vas deferens NPY induced a potentiation of the contractile effects of NA and adenosine-5-triphosphate (ATP).[47] Furthermore, NPY reduced the stimulation-

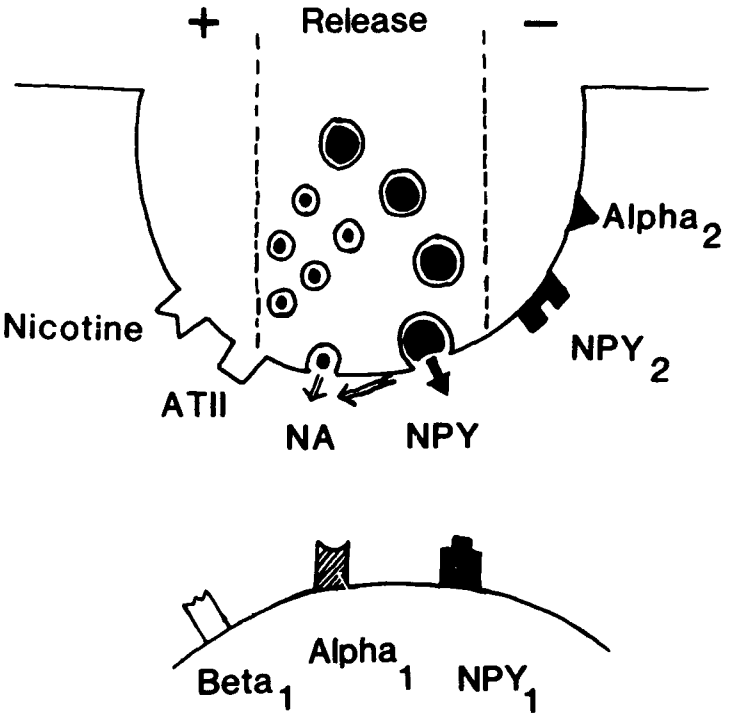

FIGURE 3. Schematical illustration of a varicosity of a sympathetic nerve containing both NA and NPY in large dense-cored vesicles and only NA in the small vesicles. At low frequency stimulation mainly small vesicles secrete their content of NA acting on postjunctional α_1-adrenoceptors to evoke vasoconstriction or β_1-adrenoceptors to increase cardiac contractility. NA also activates prejunctional α_2-adrenoceptors inhibiting release of itself and of NPY from the large vesicles. NPY is secreted upon stronger stimulation and induces vasoconstriction as well as inhibits release of NA and itself. There is some evidence that the vasoconstriction and the prejunctional effect of NPY is mediated via separate receptor mechanisms (designed NPY-1 and NPY-2, respectively). Nicotine increases basal NA and NPY secretion while angiotensin II (AT II) facilitates nerve stimulation-evoked release of NA and NPY. Due to the complex interplay between NA and NPY both at the pre- and postjunctional levels a variety of drugs which interfere with NA mechanisms will also change especially NPY release.

evoked contraction and secretion of ^{3}H-NA and selectively depressed the stimulus evoked but not the spontaneously occurring excitatory junction potentials in smooth muscle cells. Possibly this action of NPY, similarly to α_2-agonists is dependent on an inhibitory effect in part via a target upstream of the varicosities.[48]

NPY not only evokes vasoconstriction per se but also enhances the contractions of

blood vessels to a variety of agents including NA *in vitro*.[4,43] The enhancing effect of NPY is not unique but shared by other vasoconstrictors like serotonin and characterized by being more pronounced on larger vessels where NPY exerts minor or no contractions per se.

NPY Release in Human Cardiovascular Disease

The release of NPY upon sympathetic activation in healthy volunteers as revealed by increase in systemic plasma levels[34,35] or elevated levels in the coronary sinus upon hypoxia[33] combined with the potent, long-lasting vasoconstrictor effects of NPY in man[7] raise the possibility that NPY may be involved in the pathophysiology of human cardiovascular disorders. NPY is a potent constrictor of small human coronary vessels *in vitro*,[49] and elevated plasma levels of NPY are present in patients with heart disease such as acute myocardial infarction, angina pectoris and especially severe left heart failure.[50] The possible functional role of NPY under physiological conditions with intact noradrenergic mechanisms, as well as involvement in human vascular disorders, remains to be established with the use of specific NPY antagonists at the pre- and postjunctional levels. It seems clear, however, that many drugs commonly used in experimental studies on cardiovascular control or in treatment of hypertensive or vasopastic disorders in humans also influence NPY mechanisms.

SUMMARY

The coexistence of neuropeptide Y (NPY) with noradrenaline (NA) in perivascular nerves as well as in sympathetic nerves to muscle in the heart, spleen and vas deferens suggests a role for NPY in autonomic transmission. Sympathetic nerve stimulation or reflexogenic activation in experimental animals or man are associated with NPY release as revealed by overflow mainly upon strong activation. This difference between NPY and NA secretion may be related to the partly separate subcellular storage whereby NPY seems to be exclusively present in the large dense-cored vesicles. The NPY secretion is likely to be regulated by the local biophase concentrations of NA acting on prejunctional alpha-2-adrenoceptors since alpha-2 agonists inhibit and antagonists enhance NPY overflow, respectively. Furthermore, after NA has been depleted by reserpine, the nerve stimulation-evoked release of NPY is enhanced leading to a progressive depletion of tissue content of NPY. Exogenous NPY binds to both pre- and postjunctional receptors, inhibits NA and NPY release, enhances NA-evoked vasoconstriction and induces vasoconstriction per se. The prejunctional action of NPY which is especially noticeable in the vas deferens may serve to reduce transmitter secretion upon excessive stimulation. The long-lasting vasoconstriction evoked by sympathetic stimulation in several tissues including skeletal muscle, nasal mucosa and spleen, which remains in animals pretreated with reserpine (to deplete NA) combined with preganglionic denervation (to prevent the concomitant excessive NPY release and depletion), is mimicked by NPY and highly correlated to NPY release. Under these circumstances the NPY content in the local venous effluent reaches levels at which exogenous NPY evokes vasoconstriction.

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Regulation of Neuropeptide Y Gene Expression in Rat Brain[a]

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INTRODUCTION

Neuropeptide Y (NPY) is found in several rat brain regions including caudate-putamen and cerebral cortex.[1,24] NPY-like immunoreactivity in rat brain appears as one single 36-amino acid peptide.[1] Both complementary DNA (cDNA) and genomic clones have been isolated for rat NPY and used to determine the regional distribution of NPY mRNA in the rat brain.[2,13,19] NPY mRNA containing cellbodies in rat brain has also been demonstrated using *in situ* hybridization.[7,21]

In this report *in situ* hybridization and RNA blot analysis of NPY mRNA expression[d] in rat brain will be discussed and the findings compared with NPY mRNA expression in the human brain. Transsynaptic regulation of rat NPY mRNA expression by midbrain dopamine neurons is described using a unilateral 6-hydroxydopamine-induced dopamine deafferentation of the ipsilateral forebrain. For comparison, the effects of a dopamine deafferentation on preprosomatostatin (SOM), preprocholecystokinin (CCK), preprotachykinin (PPT) and glutamic acid decarboxylase (GAD; a marker of γ-amino buturic acid (GABA) neurons) mRNA expression, have also been studied. The results show that midbrain dopamine neurons differentially control expression of NPY as well as SOM, PPT, and GAD mRNA expression in a complex fashion.

METHODOLOGICAL ASPECTS

Male Sprague-Dawley rats (b.w. 150 g) were used, either naive or unilaterally injected with 4 μl of 6-hydroxydopamine (2 mg/ml in 0.02% ascorbic acid in saline) or vehicle alone into the left ventral tegmental area containing the axons leaving the midbrain dopamine neurons.[11] A unilateral lesion of ascending dopamine neurons by 6-hydroxydopamine injection in the ventral tegmental area induced dopamine deafferentation of

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[d]For simplicity, the level of different mRNAs seen after *in situ* hybridization histochemistry and RNA blot analysis is referred to as the level of mRNA expression. However, it should be pointed out that both techniques measure the steady-state level of mRNA, which may not directly reflect the level of mRNA expression, due to the possible presence of posttranscriptional controls.

several ipsilateral brain regions including caudate-putamen[11] and frontoparietal cortex.[12] The elimination of tyrosine hydroxylase (TH) mRNA hybridization in ventral tegmental area and substantia nigra ipsilateral to the 6-hydroxydopamine injection were used as control of successful lesions.

In Situ *Hybridization*

After cryostat sectioning, the tissues were fixed in 10% formalin in phosphate buffered saline for 30 min, rinsed twice for 4 min in PBS, and delipidated in graded series of ethanol, including a 5-min incubation with chloroform. The sections were then air-dried. The hybridization cocktail contained 50% formamide, 4× SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 1 × Denhardts solution, 1% Sarcosyl, 0.02M $NaPO_4$ (pH 7.0), 10% dextransulphate 0.5 mg/ml yeast tRNA, 0.06M DTT and 0.1 mg/ml sheared salmon sperm DNA. For NPY mRNA hybridization, a 44-mer oligonucleotide complementary to rat NPY mRNA encoding amino acid 15 to 29 of the mature NPY peptide was used.[19] For PPT mRNA hybridization, a 48-mer oligonucleotide complementary to rat PPT mRNA coding for amino acid 49 to 64, and thus complementary to α, β and γ-PPT mRNA was used.[18] For SOM mRNA hybridization, a 46-mer oligonucleotide complementary to rat SOM mRNA coding for amino acid 101 to 115 was used.[8] For CCK mRNA hybridization, a 44-mer oligonucleotide complementary to rat CCK mRNA coding for amino acid 89 to 103 was used.[5] For GAD mRNA hybridization a 48-mer oligonucleotide complementary to feline GAD mRNA coding for amino acid 389 to 405 was used.[14] For TH mRNA a 48-mer oligonucleotide complementary to rat TH mRNA encoding amino acid 445 to 492 of the TH polypeptide was used.[9] The oligonucleotides were 3′-end labeled with α-^{35}S-dATP using terminal deoxyribonucleotidyl transferase (International Biotech. Inc., New Haven, CT) to a specific activity of approximately 5 × 10^8 cpm/mg. The labelled probes were purified on a Nensorb column (DuPont, Wilmington, DE) prior to use. Following hybridization for 16 h at 42°C, the sections were rinsed 5 times for 15 min in 1 × SSC at 55°C. Finally, the sections were rinsed in autoclaved water for 5 min, and then dehydrated through a series of graded alcohol and air-dried. The sections were exposed to X-ray film (Amersham β-max) for 7–25 days and to photographic emulsion (Kodak NTB2) for 10–28 days. After having been developed, the slides were stained with cresyl violet followed by microscopic analysis. As a control for the specificity of the hybridization, we performed *in situ* hybridization with cDNA probes which revealed a similar hybridization pattern as the one described here. In addition, in caudate-putamen and frontoparietal cortex incubation with the TH oligonucleotide probe served as a negative control.

Neuron Counting and Computerized Image Analysis

The numerical density of hybridization positive neurons was analyzed using a Leitz Ortholux microscope at 313× magnification. Hybridization positive neurons were defined as those expressing more than 10 grains per neuron (well above background density of grains). The number of grains per neuron and the size of NPY, SOM, CCK and PPT mRNA positive neurons were calculated using a Crystal image analysis processor (Quantel Ltd, UK). For this purpose, the microscope was equipped with a Panasonic WV 1500 TV camera to provide a video signal for the image analysis. However, over some neurons analyzed the high density of grains made a direct numerical count futile. Instead, the total area of grains, also including all fused grains was divided with a standard grain area obtained from measurements of unfused grains over neurons. The area of a standard

grain was arbitrarily defined as the mean area of a few small grains measured over a hybridization positive cell.

Northern Blot Analysis

Brains from one group of animals were dissected directly after decapitation and used to prepare RNA from frontoparietal cortex and caudate-putamen. The tissue was dissected to fit with that used for *in situ* hybridization. Dissected brain tissue from fronto-parietal cortex and caudate-putamen were homogenized in 4 M guanidine isothiocyanate, 0.1 M β-mercaptoethanol, 0.025 M sodium citrate, pH 7.0 and analyzed for the level of NPY mRNA by RNA blot analysis. The homogenate was mounted on top of 4 ml cushion of 5.7 M CsCl in 0.025 M sodium citrate at pH 5.5 and centrifuged at 15°C in a Beckman SW41 rotor for 16 to 24 hours. The indicated amounts of total or polyadenylated RNA were separated on 1% agarose gels containing 0.7% formaldehyde followed by transfer to nitrocellulose filters. The filters were then hybridized to nick-translated cDNA probes labelled with α-^{32}P-dCTP to a specific activity of approximately 5×10^8 cpm/mg. The NPY cDNA prob used was a 287 bp Xba I-Ava I fragment containing the second exon of the NPY gene.[19] The filters were washed at high stringency (0.1 × SSC, 0.1% SDS at 54°C) and exposed to Kodak XAR-5 films. The same filters as used for NPY hybridization, were boiled for 5 min in 1% glycerol and rehybridized to a nick-translated 1.5 kb PstI fragment from a mouse α-actin cDNA clone.[23] Appropriate exposures, *i.e.*, exposures that allowed measurements of the hybridization signal in a linear interval of all autoradiograms were quantified using a Shimadzu CS-9000 densitometer. The levels of NPY mRNA were then normalized to the levels of actin mRNA.

RESULTS

NPY mRNA Expression in Control and Dopamine Deafferentated Caudate-Putamen and Frontoparietal Cortex

NPY mRNA hybridizations[21] revealed a specific labeling in several discrete brain regions, including septal nuclei, basal forebrain and hypothalamic nuclei (FIG. 1). Microscopic examination of emulsion dipped tissue sections showed dense clusters of grains over neurons throughout caudate-putamen, nucleus accumbens and cerebral cortex (FIG. 2). Less than 5% of neurons were positive in these regions. The labeling was present over all cortical layers, with a concentration over layers V and VI. A two-times higher numerical density of NPY mRNA positive neurons was seen in frontoparietal cortex compared with caudate-putamen. Furthermore, the number of grains per NPY mRNA positive neuron in frontoparietal cortex was 47% higher than in caudate-putamen.

In the dopamine deafferentated side the numerical density of NPY mRNA positive neurons in caudate-putamen increased approximately two-fold compared with the intact, contralateral caudate-putamen. The number of grains per NPY mRNA positive neuron in caudate-putamen was, however, not changed when comparing the deafferentated caudate-putamen with the contralateral caudate-putamen. In the dopamine deafferented frontoparietal cortex, the numerical density of NPY mRNA positive neurons was less than half of that in the intact side. The number of grains per NPY mRNA positive neuron in frontoparietal cortex was, as in caudate-putamen, not changed when the two sides were compared.

RNA blot analysis showed the presence of a single 0.8 kb NPY mRNA in caudate-

putamen and frontoparietal cortex (FIG. 3). Furthermore, the RNA blot analysis, originally reported here, showed an increase with 62% in the level of NPY mRNA in the dopamine deafferented caudate-putamen and a decrease with 79% in the deafferented frontoparietal cortex (FIG. 3) compared with corresponding regions in sham-operated animals. The results from the RNA blots are in agreement with the *in situ* hybridization data.

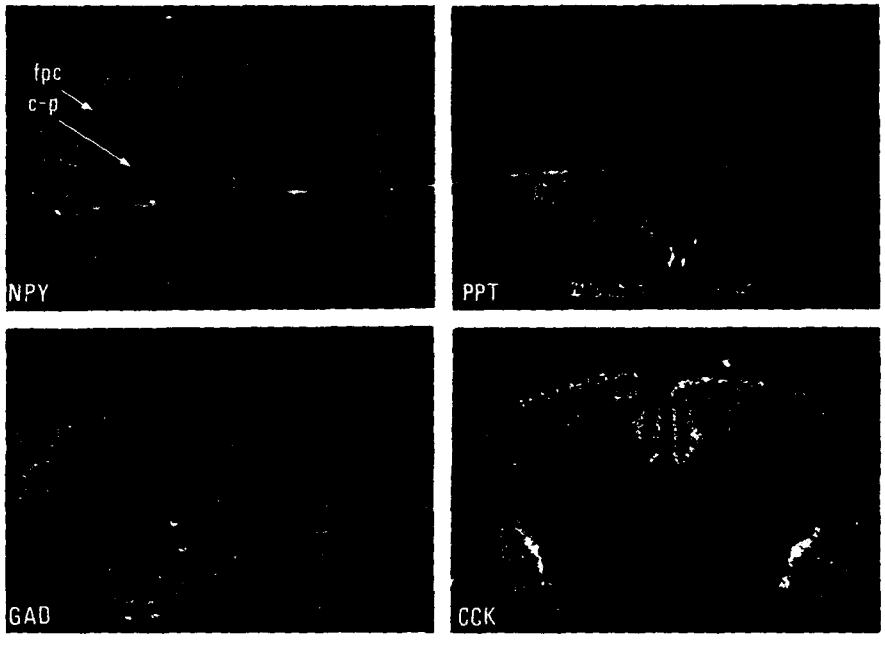

FIGURE 1. *Upper left panel* shows a low magnification dark-field photomicrograph of NPY mRNA *in situ* hybridization histochemistry in caudate-putamen and frontoparietal cortex, after a unilateral 6-hydroxydopamine-induced lesion of midbrain dopamine neurons. The tissue was hybridized to a oligonucleotide specific for rat NPY mRNA and exposed to a photographic film. PPT, GAD and CCK mRNA hybridizations from adjacent tissue sections are shown for comparison. Abbreviations: fpc = frontoparietal cortex, c-p = caudate-putamen, CO = contralateral side, IP = ipsilateral side. (Modified from REFERENCES 20 and 21.)

SOM, CCK, PPT and GAD mRNA Expression in Control and Dopamine Deafferentated Caudate-Putamen and Frontoparietal Cortex

SOM mRNA hybridizations[21] showed a specific labeling with a distribution very similar to that of NPY. However, in contrast to NPY the number of grains per SOM mRNA positive neuron in frontoparietal cortex was similar to that in caudate-putamen. The regulation of SOM mRNA expression was in most cases similarly affected by the dopamine deafferentation as in the case of NPY mRNA expression. However, in contrast to NPY, the grain density over SOM mRNA positive neurons decreased (24%) in frontoparietal cortex, while the numerical density of SOM mRNA positive neurons was unchanged.

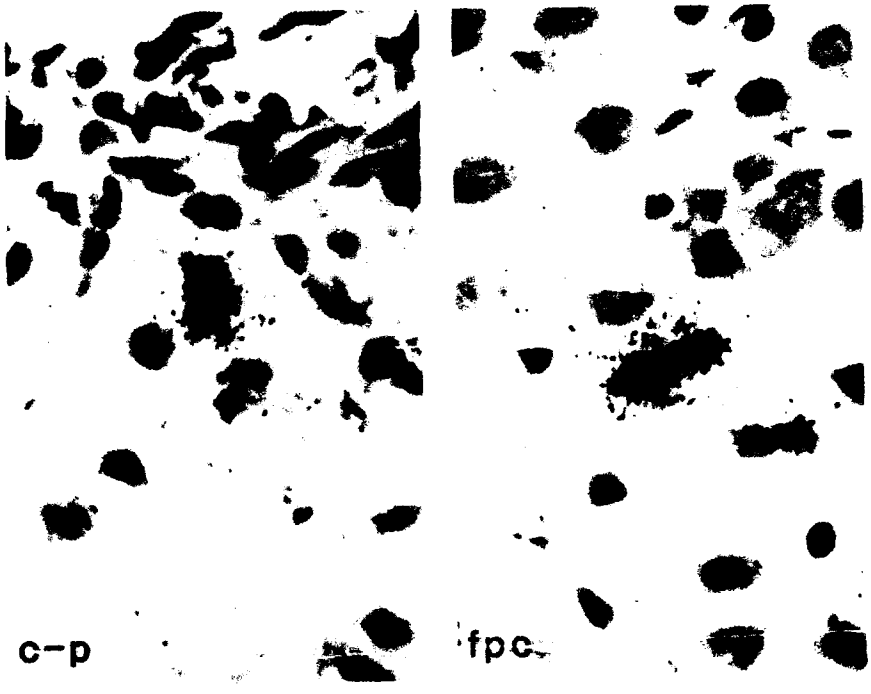

FIGURE 2. High magnification bright-field emulsion photomicrographs of NPY mRNA positive neurons in rat frontoparietal cortex and caudate-putamen. The neurons shown are representative, and selected from the same section as shown in the upper left panel of FIGURE 1. Note that the cortical neuron is larger and more intensely labeled. Abbreviations as in FIGURE 1.

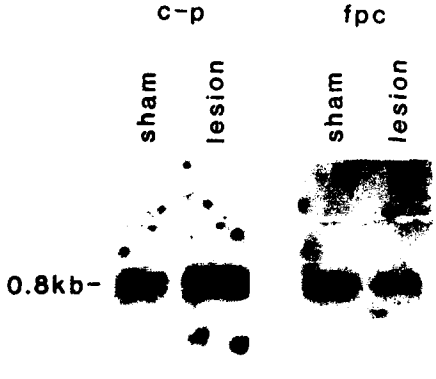

FIGURE 3. Expression of NPY mRNA in caudate-putamen and frontoparietal cortex (ipsilateral to injections) in 6-hydroxydopamine-injected and sham-injected animals. Total RNA (35 μg/slot) from a pool of 3 to 5 animals was electrophoresed in a formaldehyde containing agarose gel followed by transfer to nitrocellulose filters. The filters were hybridized to a ^{32}P-labelled 287 bp Xba I-Ava I cDNA probe for rat NPY mRNA. The filters were washed at high stringency followed by autoradiography. Note the increased level of NPY mRNA in ipsilateral caudate-putamen and the decreased level in ipsilateral frontoparietal cortex of lesioned animals. All values determined from the films were normalized to the level of actin. Abbreviations as in FIGURE 1.

CCK mRNA hybridizations[21] (FIG. 1) showed an intense two-layer labeling in the cerebral cortex with much higher numerical density of labeled neurons than after hybridization to NPY or SOM probes. However, no hybridization signal was detected in either the lesioned or the intact caudate-putamen. The numerical density of CCK mRNA positive neurons in frontoparietal cortex, as well as the number of grains over CCK mRNA positive neurons, were not changed after the dopamine deafferentation.

PPT mRNA hybridizations[21] (FIG. 1) showed an apparent homogenous labeling in caudate-putamen and nucleus accumbens with a few intense dots. Microscopic examination of the sections showed that the apparent homogeneous signal consisted of many relatively weakly labeled neurons, while the intense dots represented sparsely distributed, intensively labeled neurons. 40 to 60% of all neurons were PPT mRNA positive in caudate-putamen. In the cerebral cortex the PPT probe showed labeling only over very few cells. The numerical density of PPT mRNA positive neurons in the dopamine deafferented caudate-putamen decreased to one fifth (fivefold) compared with the intact caudate-putamen. Furthermore, the number of grains over PPT mRNA positive neurons in the deafferented caudate-putamen decreased by one half compared with the intact caudate-putamen.

GAD mRNA hybridizations[20] (FIG. 1) revealed two populations of GAD mRNA positive neurons in caudate-putamen. The majority of neurons in this region were GAD mRNA positive and of the hybridization positive neurons the majority showed a weak labeling, while only less than 10% of GAD mRNA positive neurons showed intense labeling. The numerical density of GAD mRNA hybridization positive neurons was lower in frontoparietal cortex but the intensity of hybridization signal (grain density) was higher over individual cortical neurons compared to that in caudate-putamen. The dopamine deafferentation of caudate-putamen induced an increase in the number of intensively labelled neurons with 46% as compared with the unlesioned side. However, the total number of positive neurons was similar in both sides. The number of grains per neuron over the intensely labelled neurons was similar in both sides. In frontoparietal cortex, the dopamine deafferentation reduced the number of neurons expressing high levels of GAD mRNA to less than half the number seen in the intact side. The remaining highly expressing neurons showed, however, similar amounts of grains per neuron as the intensely labelled neurons in the intact side. Thus, GAD mRNA expression was changed in similar directions as NPY mRNA expression, by the dopamine deafferentation, both in caudate-putamen and in frontoparietal cortex. Furthermore, similar to NPY mRNA, GAD mRNA expression seemed to be affected in a subpopulation of hybridization positive neurons in both caudate-putamen and frontoparietal cortex.

Neuronal Dimensions

Mean cross-section areas of the NPY mRNA positive neurons were also studied using a computerized image analysis system. In caudate-putamen, the mean cross-section area of the NPY mRNA positive neurons was 234 μm^2 (SEM = 17; n = 20) in the intact side and 200 μm^2 (SEM = 16; n = 20) in the lesioned side. In frontoparietal, cortex NPY mRNA positive neurons were 274 μm^2 (SEM = 20; n = 20) and 274 μm^2 (SEM = 17; n = 18) in the intact and lesioned side, respectively.

DISCUSSION

In situ hybridization and RNA blot analysis were used to study expression of NPY mRNA in rat brain. Expression of SOM, CCK, PPT and GAD mRNA expression was

studied for comparison.[20,21] To evaluate transsynaptic regulation of mRNA expression, rats were unilaterally dopamine deafferented by an injection of 6-hydroxydopamine into the mesencephalon and mRNA levels were compared with levels in sham-injected controls and in naive animals. Specific labeling was found in several brain regions. NPY and SOM mRNA showed a similar distribution in the rostral part of telencephalon, with scattered intensely labeled neurons in cerebral cortex and caudate-putamen, as well as in adjacent brain regions. The hybridization patterns of PPT and CCK mRNA were clearly different from those of NPY and SOM mRNA. CCK mRNA hybridization in the rostral telencephalon was restricted to the cerebral cortex and showed labeling over two distinct layers. At low magnification the overall PPT mRNA labeling was intense in caudate-putamen, nucleus accumbens and olfactory tubercles, while in the cerebral cortex only very few cells are positive. In contrast to both NPY and SOM mRNA positive neurons, most individual PPT mRNA positive neurons in caudate-putamen were weakly labeled, although a few scattered intensely labeled neurons were also found. A similar normal distribution of NPY, SOM, CCK, PPT and GAD mRNA expression in rat brain also has been reported by others (for REFS. see 20,21). We have also investigated the distribution of NPY mRNA expression in human brain,[4] and NPY mRNA positive neurons were found with a similar distribution as in rat brain. However, in human brain the numerical density of NPY mRNA expressing neurons was much lower in cortex cerebri compared to in caudate nucleus and putamen, while in rat brain the numerical density of NPY mRNA positive neurons was higher in cerebral cortex compared to in caudate-putamen.[21]

The expression of NPY and SOM mRNAs appears to be similarly, but not identically regulated by dopamine. In frontoparietal cortex ipsilateral to the dopamine lesioned side, the numerical density of NPY mRNA positive neurons decreased by more than one half (FIG. 4), whereas the number of SOM mRNA positive neurons was not changed. The grain density over labeled neurons in cerebral cortex, on the other hand, was not changed over NPY expressing neurons (FIG. 4), but decreased over SOM expressing neurons. Thus, midbrain dopamine neurons appear to control neuronal expression of NPY and SOM mRNA by a tonic upregulation in frontoparietal (somatosensory) cerebral cortex. NPY mRNA expression in cortex appears to be regulated by dopamine at the level of the number of neurons expressing NPY mRNA. Alternatively, dopamine may drastically change the level of NPY mRNA expression, and bring NPY mRNA above detection limit, in a subpopulation of cortical neurons, that without dopamine, produce very low NPY mRNA levels.

In caudate-putamen, the dopamine deafferentation increased the number of NPY mRNA (FIG. 4) expressing neurons whereas the grain density over neurons was not changed in either case and the same response was seen for SOM mRNA expression. This suggests that dopamine exerts a tonic downregulation of NPY and SOM mRNA expression in the intact caudate-putamen. Interestingly, the image analysis revealed that the mean size of NPY mRNA positive neurons was decreased in the ipsilateral caudate-putamen. No such change was seen in the SOM mRNA positive neurons. Since the numerical density of NPY mRNA positive neurons was doubled in the ipsilateral caudate-putamen compared with the contralateral side, it is possible that these new NPY mRNA expressing neurons represent a population of neurons different from those in the intact caudate-putamen. GAD mRNA is expressed in different neuronal populations in caudate-putamen, and the dopamine deafferentation increased expression of GAD mRNA in one subpopulation of medium size neurons[20] (mean surface area of 163 μm^2). A simple calculation (mean area of NPY mRNA positive neurons in ipsilateral caudate-putamen is 200 μm^2 and in contralateral caudate-putamen 234 μm^2; give $(234\ \mu m^2 + X)/2 = 200\ \mu m^2$; $X = 166\ \mu m^2$) indicates that the new NPY mRNA positive neurons have a mean surface area of around 166 μm^2, *i.e.*, similar to the size of the GAD mRNA expressing neurons that appear in the dopamine deafferented caudate-putamen. Moreover, GAD and

NPY have been shown to coexist in brain neurons.[10] It is therefore possible that ascending dopamine neurons have a tonic inhibitory effect on expression of both NPY and GAD mRNA in a population of caudate-putamen neurons which use both GABA and NPY as neurotransmitters/neuromodulators.

The possibility of dopamine regulation of NPY expression in neurons of caudate-putamen is also strengthened by the findings of synaptic dopaminergic input on NPY-immunoreactive neurons in this region.[17] Furthermore, a unilateral lesion of ascending dopamine neurons with 6-hydroxydopamine increases the number of NPY-immunoreactive neurons in the ipsilateral caudate-putamen.[15] The possibility that dopamine receptors

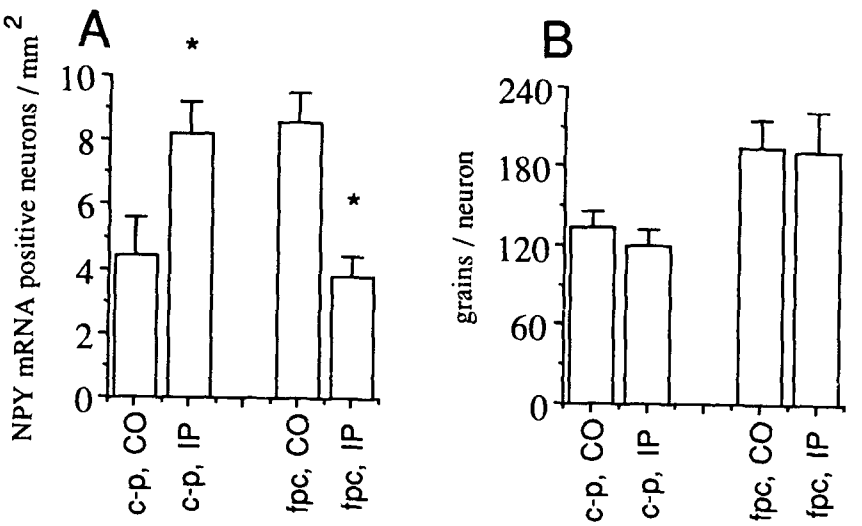

FIGURE 4. **(A)** Numerical density (neuron/μm^2) of NPY mRNA positive neurons in caudate-putamen and frontoparietal cortex contra- and ipsilateral to a lesion of ascending mesencephalic dopamine neurons. Values counted from coronal sections at the rostro-caudal level of Bregma + 1.0 to 1.6. The positive neurons counted for each animal (over 1 mm^2 in total, in two-tissue sections) corresponded to those around the tips of the *arrows* as indicated in FIGURE 1. Values are mean ± SEM (n = 5). * indicates difference between contra- and ipsilateral side ($p < 0.05$, Student two-tailed *t* test for paired samples). **(B)** Density of silver grains over single representative neurons in caudate-putamen and frontoparietal cortex contra- and ipsilateral to a lesion of ascending mesencephalic dopamine neurons. Values were calculated using a computerized image analysis system and the values represent number of grains per neuron in mean ± SEM (n = 5). Sections with appropriate exposure time were used for each probe. Abbreviations as in FIGURE 1.

regulate peptide levels differentially has been studied in pharmacological experiments. Dopamine D_2 receptor antagonists have been shown to decrease the number of NPY-immunoreactive neurons in caudate-putamen, whereas a D_1 receptor antagonist causes an increase in the number of NPY-immunoreactive neurons.[3,16] It is therefore possible that the increased level of NPY and SOM mRNA that we have observed in caudate-putamen after a dopamine deafferentation is caused by a decreased stimulation of dopamine D_1 receptors.

In contrast to NPY mRNA regulation, the dopamine deafferention of caudate-putamen clearly decreased PPT mRNA expression, suggesting a tonic stimulatory effect by striatal

dopamine on PPT expression in caudate-putamen. These data are in agreement with earlier findings where a unilateral dopamine deafferentation have been shown to inhibit PPT mRNA expression in caudate-putamen.[27]

No evidence was found for a change in cortical CCK mRNA expression by the dopamine deafferentation. This finding is in agreement with recent data from Savasta and collaborators,[25] who also did not find an effect on CCK mRNA expression in the somatosensory cortex after a unilateral dopamine lesion with 6-hydroxydopamine. The lack of a response by cortical CCK mRNA expression to the dopamine deafferentation, is in contrast to the change in SOM, NPY and GAD mRNA expression. This is probably due to the fact that cortical CCK and cortical NPY/SOM/GAD are located in different neuron populations.[10,26]

Alterations in neuropeptide levels in the cerebral cortex have also been studied using other lesions or treatments. Ibotenic acid induced lesion of rat nucleus basalis, containing cholinergic afferents to the cerebral cortex, results in an increased density of NPY- and SOM-immunoreactive fibers in the parietal cortex,[6] indicating a regulation opposite to that after a dopamine deafferentation. Furthermore, a systemic injection of kainic acid in rat that decrease seizure threshold, change GAD enzyme activity in similar direction as changes in tissue levels of NPY and SOM.[22] This finding suggests that co-existing GABA/SOM/NPY in cortical neurons,[10] may be co-regulated. A similar correlation can be suggested when comparing the results from NPY mRNA hybridizations in frontoparietal cortex of dopamine deafferented rats with GAD mRNA hybridizations in the same region. The numerical density of NPY and GAD mRNA hybridization positive neurons both decrease with 55–57%,[20,21] indicating dopaminergic regulation of a subpopulation of cortical neurons that possibly use both NPY and GABA as neuromodulators/neurotransmitters.

SUMMARY AND CONCLUSIONS

NPY mRNA expression was studied in rat brain using *in situ* hybridization and RNA blot analysis. Transsynaptic regulation of NPY gene expression was specifically studied in caudate-putamen and frontoparietal (somatosensory) cortex of rats with unilateral lesion of midbrain dopamine neurons and in sham-injected animals. NPY mRNA expression in these two brain regions and the regulation by midbrain dopamine neurons were compared with that of SOM, PPT, CCK and GAD mRNA expression. Neurons expressing NPY and SOM mRNA showed a similar distribution and the expression of both NPY and SOM appears to be regulated by dopamine in a similar fashion. Following a unilateral dopamine deafferentation, the numerical density of both NPY and SOM mRNA expressing neurons almost doubled in the lesioned rat caudate-putamen with no change in the average grain density over positive neurons. Hence, in the intact caudate-putamen dopamine appears to normally suppress expression of these two neuropeptide genes. An activation of both NPY and SOM mRNA expression in many non- or low-expressing neurons is seen when the level of dopamine is decreased. In the frontoparietal cortex, on the other hand, dopamine appears to stimulate NPY and SOM gene expression. RNA blot analysis shows clear-cut changes of NPY mRNA levels in both caudate-putamen and frontoparietal cortex consistent with the changes observed using *in situ* hybridization. No evidence was found for a change in CCK mRNA expression by the dopamine deafferentation, while PPT mRNA expression decreased in the deafferented caudate-putamen. Consequently, dopamine exerts dissimilar effects on the expression of different neuropeptide genes, that in turn do not respond in the same way in different brain regions. Indirect evidence is also presented indicating that dopamine regulates NPY mRNA ex-

pression in a subpopulation of neurons that possibly also express GAD mRNA, both in caudate-putamen and in frontoparietal cortex.

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Neuropeptide Y in Cortex and Striatum

Ultrastructural Distribution and Coexistence with Classical Neurotransmitters and Neuropeptides[a]

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid polypeptide that is structurally similar to the pancreatic polypeptide (PP) and the intestinal peptide YY (PYY).[1] Radioimmunoassay and immunocytochemical techniques have revealed a wide distribution of NPY in the central, peripheral and enteric nervous systems in species ranging from insects to humans.[2] Consistent with its distribution, many physiological functions are modulated by NPY. These include regulations of the cardiovasculature, kidney and neuroendocrine function, sympathetic neurotransmission, and memory processing (reviewed elsewhere in this volume). NPY may modulate basal ganglia function, since it can increase the turnover of dopamine in the striatum.[3] Recent studies suggest that NPY plays important roles in the perinatal development of cerebral cortex. This idea is based on observations that neurons containing this peptide occur transiently in time and place where the thalamic afferents wait before beginning to innervate the developing cortex.[4,5]

The diversity of NPY action may depend, in part, on the intracellular mechanisms through which NPY interact with various transmitters. Thus, this paper will briefly review earlier studies describing the distribution and coexistence of NPY with other transmitters and peptides in the striatum and cortex. The anatomical circuitry involving NPY-neurons, *i.e.*, the types of input, targets and the intrinsic versus efferent connectivities, also are expected to dictate NPY function. The focus of this updated review is to describe the cellular and ultrastructural basis for intra- and intercellular relationship among neurons containing NPY, GABA and catecholamines in the two regions. Portions of the review are based on our recent studies in the cerebral cortex and striatum of rat and cat brains.[6,7]

Anatomical Methods Used to Identify NPY-Neurons

In 1983, Vincent *et al.* used antisera directed against the avian pancreatic polypeptide (anti-aPP) to show that striatal neurons in rat brains exhibiting high levels of NADPH-

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diaphorase activity contained both somatostatin- and aPP-like peptides.[8,9] Succeeding studies confirmed that these neurons actually contained NPY, since aPP-immunoreactivity could be abolished by preadsorption of the antiserum with NPY [9] and because the neurons could be stained dually for aPP and NPY within single sections.[11] These results indicate that all PP-immunoreactive neurons probably contain NPY. However, a possibility remains that the anti PP may fail to recognize all NPY-neurons.

NPY probably is synthesized by the neurons containing NPY, since *in situ* hybridization studies show close resemblance in the regional distribution of neurons containing high levels of NPY-mRNA and aPP-immunoreactivity.[12,13] However, the identification of NPY-mRNA and NPY-immunoreactivity within single neurons would be required to confirm this point.

We used a goat antiserum directed against the carboxy-terminal hexapeptide of aPP to label the NPY-neurons by the peroxidase-antiperoxidase (PAP) immunocytochemical method[14] within free-floating, aldehyde-fixed Vibratome sections of rat forebrain[15] and cat visual cortex. The antiserum was generously provided to us by Dr. O'Donohue's laboratory at the National Institutes of Health, Bethesda, MD and has been shown to specifically recognize NPY within brain tissue.[11,12,16] After the PAP-labeling for NPY,the rat brain sections were labeled for GABA using a rat antiserum directed against a protein-conjugated form of GABA. This antiserum was generously provided to us by Dr. Andrew C. Towle, Cornell University Medical College and has been characterized for its specificity.[17] The antigenic sites for anti-GABA then were recognized by the immunoautoradiographic technique.[18] Alternatively, the rat brain sections labeled for NPY by the PAP method were labeled for catecholamines by using a rabbit antiserum directed against the catecholamine-synthesizing enzyme, tyrosine hydroxylase (TH) and applying the immunoautoradiographic technique. These sections were analyzed for the distribution of immunoreactive neurons and processes under light and electron microscopy. Further details of methods have been published.[6,7]

The Morphological Characteristics and Distribution of NPY-Neurons

Neurons immunolabeled by the anti-aPP antiserum show features that are in close accord with previous descriptions.[8-10,16,19] Within the striatum, the anti-aPP antiserum labels cell bodies that are sparsely distributed, oblong, rectangular or triangular in shape and 15 to 25 μm in diameter. The density of NPY-neurons is higher ventrally than dorsally. Two to three processes, presumably dendrites, emanate from these cell bodies. They are approximately 1 μm wide near the cell body, taper distally and are varicose but not spiny. Within the cerebral cortex, neurons with similar morphology (FIG. 1A,B) occur in laminae II/III and V/VI, and rarely within laminae I or IV. In addition, NPY-immunoreactive neurons also occur near and within the white matter. They usually are fusiform in the tangential orientation and show dendrites that course longitudinally along the orientation of fibers in the white matter.

The ultrastructural features of neurons containing NPY are similar in the dorsal and ventral striatum and in the cerebral cortex. Immunoperoxidase reaction product reflecting the presence of NPY is diffusely distributed throughout the neuronal perikarya. Perikarya contain large, indented nuclei and prominent stacks of rough endoplasmic reticulum (FIG. 1C) and Golgi apparatus. These morphological features frequently are associated with cells undergoing rapid turnover of peptides.[20] The plasma membranes have irregular contours, sparse distributions of axo-somatic contacts and are largely surrounded by processes and cell bodies of glia.

Fibers immunoreactive for NPY appear varicose by light microscopy (FIG. 2). They give rise to fine short collaterals at regular intervals, and have main axes that extend long

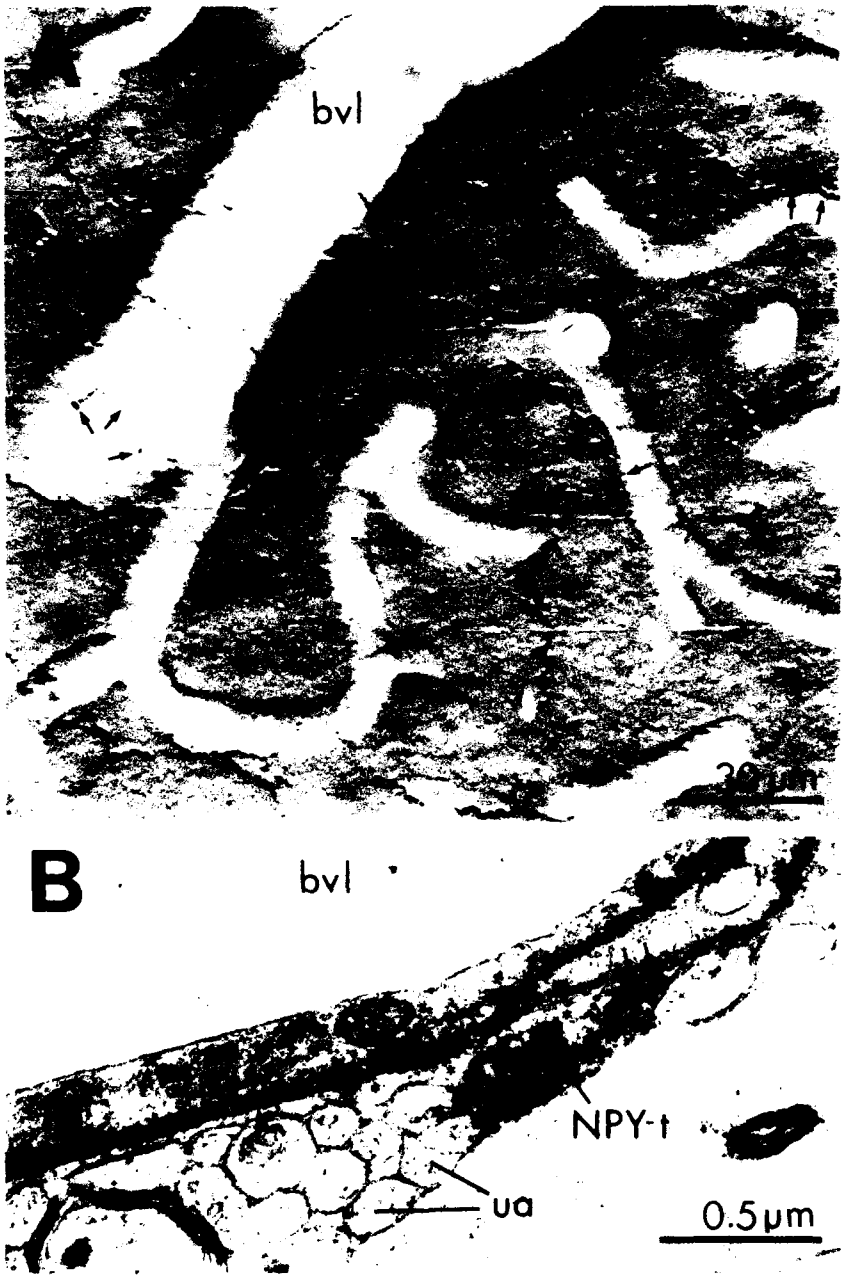
FIGURE 1. The relation of NPY-neurons to blood vessels. Light micrographs in **(A)** and **(B)** show NPY-immunoreactive cell bodies and processes (*arrowheads*, probably dendrites) in the cat cortex that are in close proximity to blood vessels. Intensely immunoreactive fibers (*small arrows*) are varicose and course over blood vessels. The electron micrograph in **(C)** shows the peroxidase localization, reflecting NPY-like immunoreactivity, within a neuronal perikaryon (NPY-n over nucleus) in the dorsal striatum of a rat brain. The nucleus of the labeled cell is indented. Prominent stacks of rough endoplasmic reticulum are evident near the plasma membrane that is juxtaposed to a glial cell (GC over nucleus), which, in turn, is adjacent to a blood vessel. E = nucleus of an endothelial cell. *Asterisks* in A–C = blood vessel lumens. (FIG. 1C modified from REF. 6.)

distances. For example, fibers greater than 100 μm within the cortex and striatum of rat brains and greater than 1 mm in the monkey cortex have been reported.[10,16] These fibers generally course tangentially within the superficial and deeper laminae, and radially in the laminae in between. During perinatal development, some of the NPY neurons in the deeper layers of cat visual cortex are reported to possess very long axons that course radially from the white matter to layer I (greater than 3 mm).[4] Others in the superficial layers send axons that proceed towards the white matter. These transiently occurring neurons possess axons that form loops, and are thus called "axonal loop cells."[4]

Electron microscopy reveals that NPY-immunoreactivity is much more abundant within axons than in cell bodies or dendrites (FIG. 3). NPY-immunoreactive axons can be followed to traverse in and out of the ultrathin section and make numerous contacts within a small region. These contacts resemble symmetric (presumably inhibitory[21]) synaptic junctions. These contacts are very narrow and thus do not persist beyond one or two serial ultrathin sections (ca. 50 nm thick). Most portions of NPY-axons appear terminal-like in that they are packed with small clear vesicles that are intensely rimmed by the immunoperoxidase reaction product. Occasionally, these small vesicles are found to coexist with a few larger vesicles that are immunoreactive within the core (not shown in Fig. 3). Within the ventral striatum, we have also observed terminals containing numerous large vesicles that are immunoreactive only within cores. Small clear vesicles that are nonimmunoreactive coexist with these immunoreactive dense-core vesicles. By using dual immunocytochemical techniques, we have shown that at least some of these terminals are catecholaminergic (FIG. 3). Thus, although all NPY neurons may be intrinsic,[22] not all NPY fibers originate from these intrinsic neurons. These terminals may be noradrenergic or adrenergic rather than dopaminergic, since terminals with this type of intracellular distribution for NPY-immunoreactivity do not occur in the dorsal striatal area recognized to receive heavy dopaminergic innervation, but have been observed in the sensory neocortex[10] and nucleus of the solitary tract[23] which contain more of the nondopaminergic catecholaminergic fibers. The afferents are likely to arise in the brainstem areas that are known to contain both NPY and norepinephrine or epinephrine.[24-27]

The Relation of NPY-Neurons to Blood Vessels

Along the pial surface, previous light microscopic studies have described intimate relationships between blood vessels and catecholaminergic fibers arising from the superior cervical ganglion and containing NPY[28] (also reviewed in this volume). This relationship is interesting, because NPY has been shown to exert potent vasoconstricting effects that are calcium-dependent.[28] We have examined the possible relationship between NPY-neurons and blood vessels within the cortical grey matter by light and electron microscopy. For this purpose, we postfixed the sections that were immunocytochemically labeled for NPY with 2% osmium tetroxide for 1 hour to allow better visualization of blood vessels by light microscopy. Sections prepared by this method show many NPY-immunoreactive cell bodies and dendritic processes that are in close proximity to capillaries and larger blood vessels (FIG. 1A,B). However, ultrastructural examination of sections immunolabeled for NPY shows that the NPY-neurons rarely, if ever, form direct contact with the basement membrane of pericytes or endothelial cells of the blood vessel wall. Instead, NPY-immunoreactive processes are frequently in direct contact with glial cell bodies. Glial cells, in turn, frequently contact the basement membrane of blood vessel walls (FIG. 1C). Also notable are NPY-immunoreactive varicose processes (probably axons) that appear to loop around or course along small and larger blood vessels within light microscopic preparations (FIG. 2A,B). Ultrastructural examination of NPY-immunoreactive terminals show that direct contact between these fibers and blood vessels is


bvl
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also rare. Under close scrutiny, fine glial processes interleave the space between NPY-terminals and the basement membrane of blood vessels (FIG. 2C). Glial cells have been shown to possess NPY-binding sites.[29] Thus glial processes may be involved in the transduction of NPY-mediated signals to and from blood vessel wall or circulation. In addition, brief interruptions in the glial interposition do occur (FIG. 2C). These may be sites for direct interactions between NPY-neurons and blood vessels.

Chemical Heterogeneity among NPY-Neurons

The studies of Vincent *et al.*[8] were the first to indicate chemical heterogeneity among NPY-neurons. They showed that *all* neurons in the striatum exhibiting NADPH-diaphorase activity contained immunoreactivities for both NPY-like substances and somatostatin. In contrast, some of the NPY-neurons in the cerebral cortex were NADPH-diaphorase-negative and contained lower levels of NPY-immunoreactivity.

In the following years, Hendry *et al.* used dual immunocytochemical techniques to show that the NPY-neurons in the cerebral cortex of the rat and monkey co-localize not only with somatostatin but also with glutamate decarboxylase, the GABA-synthesizing enzyme.[10,30] In fact, NPY is one of many peptides and transmitter-like substances that are now recognized to coexist with GABA.[31] More recent dual immunocytochemical studies indicate that a small population of NPY-neurons are not GABAergic but coexist with tachykinins.[32]

The NPY-neurons in the striatum have not been considered to be GABAergic. This notion was based on the knowledge that NPY-neurons are aspiny[8,9] and intrinsic,[22] while most of the GABAergic neurons in the striatum are spiny and make efferent projections.[33–35] Moreover, postmortem analysis of striatum afflicted with Huntington's disease showed drastic loss in GABAergic neurons, and at the same time, sparing of NPY-immunoreactive neurons.[36] This suggested that the NPY-neurons in the striatum were distinct from the GABAergic population of neurons. On the other hand, Bolam *et al.*[37] had reported on the existence of a population of aspiny neurons in the striatum that accumulated GABA. Thus, a possibility remained that a smaller, aspiny population of neurons in the striatum also were GABAergic, or, instead, had capacities for uptake but not the release of GABA. To clarify this point, we examined the distribution of GABA in relation to NPY at pre- and postsynaptic sites within neurons of the striatum, then compared with the distribution of GABA and NPY in the cerebral cortex.

Light microscopic examination of the sections labeled dually for GABA and NPY showed that the majority of NPY-immunoreactive cell bodies in the anterior cingulate and somatosensory areas of cerebral cortex also contained GABA-immunoreactivity, while most of the NPY-neurons in the caudate-putamen lacked GABA-immunoreactivity. How-

FIGURE 2. The relation of blood vessels to fibers with NPY-like immunoreactivity. The light micrograph in **(A)** shows NPY-immunoreactive varicose fibers in a section from the cat visual cortex that was postfixed with 2% osmium tetroxide. Some of the fibers can be seen to course along blood vessels *(double and triple small arrows)* or show varicosities near the wall of blood vessels *(single small arrows)*. *Large arrow* points to lumen of a portion of a small diameter vessel, possibly a capillary. The electron micrograph in **(B)** shows an axon containing NPY-like immunoreactivity near a blood vessel (bvl = blood vessel lumen) of the rat somatosensory cortex. The wall of the blood vessel shows basement membranes *(arrowhead pairs)* that are ensheathed by a fine glial process (*asterisks* within cytoplasm). The NPY-terminal (NPY-t) courses adjacent to the glial process, and at one point, is immediately adjacent to the basement membrane *(double arrows)*. ua = unlabeled unmyelinated axons. (FIG. 2B modified from REF. 6.)

FIGURE 3. The dual ultrastructural localization of NPY and tyrosine hydroxylase. The electron micrograph, taken from the ventral striatum of a rat, shows the ultrastructural distribution of NPY-like immunoreactivity within a singly labeled dendrite and in a terminal containing only NPY or NPY and TH. The immunoreactivity is more intense within terminal (NPY-t) than in the dendrite (NPY-d). Although most NPY-terminals show a diffuse distribution of immunoreactivity that excludes lumens of small vesicles, a select population of axons contain a large number of nonimmunoreactive vesicles admixed with large vesicles that are immunoreactive only within cores (dcv). Immunoautoradiographic labeling for tyrosine hydroxylase (silver grains) indicates that these are catecholaminergic axons (CA-t). (Modified from REF. 6.)

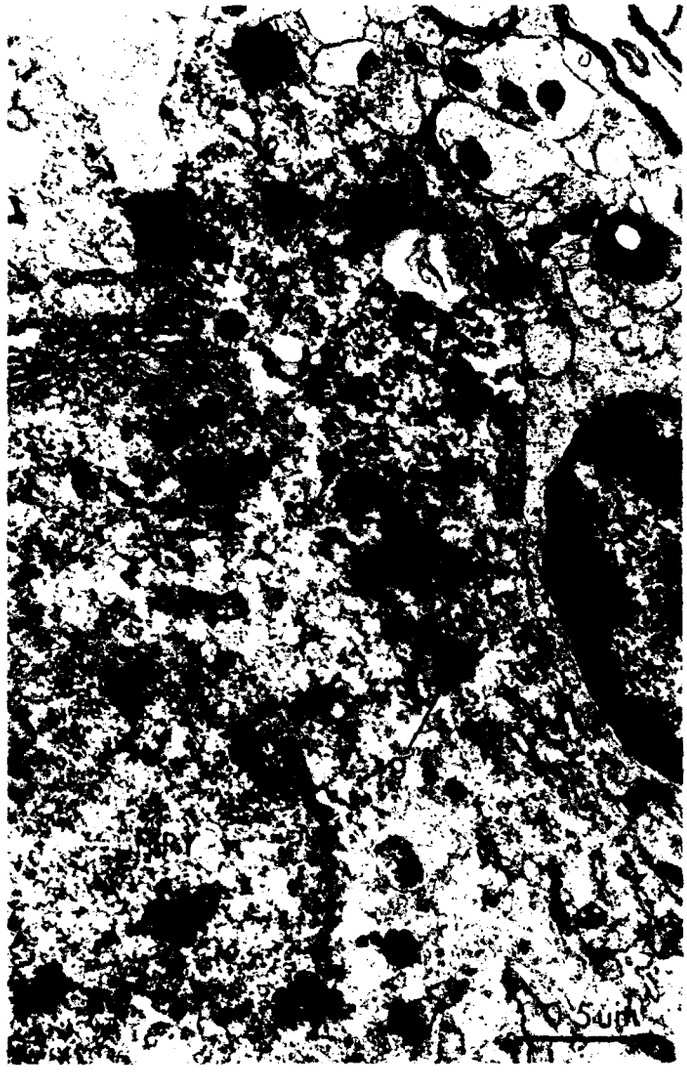

FIGURE 4. Ultrastructural localization of GABA- and NPY-immunoreactivity in a perikaryon within the infragranular layer of rat somatosensory cortex. The electron micrograph shows a diffuse distribution of peroxidase reaction product, reflecting NPY-immunoreactivity, within the cytoplasm that contains a well-developed Golgi apparatus (Ga) and dense-core vesicles (dcv). Numerous silver grains (sg), indicating GABA-immunoreactivity, overlie the cytoplasm as well as the nucleoplasm (NPY-n). The somatic plasma membrane is in contact with a glial cell (GC). *Arrowheads* point to the cellular boundary of the dually labeled neuron. (Modified from REF. 7.)

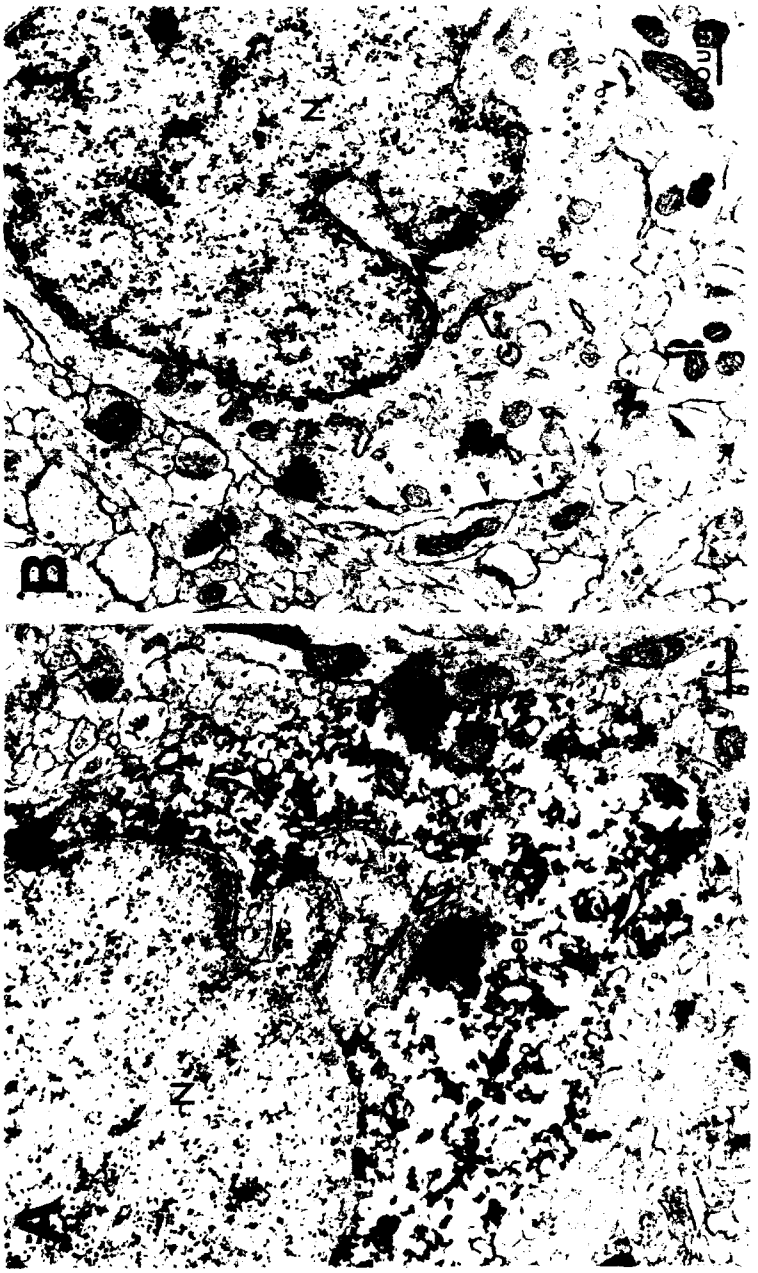

FIGURE 5. GABAergic perikarya with and without NPY-immunoreactivity in the rat striatum. The electron micrographs in **(A)** and **(B)** show silver grains reflecting GABA-immunoreactivity over the perikaryal cytoplasm. Cytoplasm of the neuron in (A) also show immunoperoxidase reaction product reflecting NPY-immunoreactivity. In both perikarya, the Golgi apparatus (G) occurs near the indentation of their nuclear envelopes *(curved arrow)*. Bar = 0.5 μm. (From Aoki and Pickel.[7] Reprinted by permission from the *Journal of Neuroscience*.)

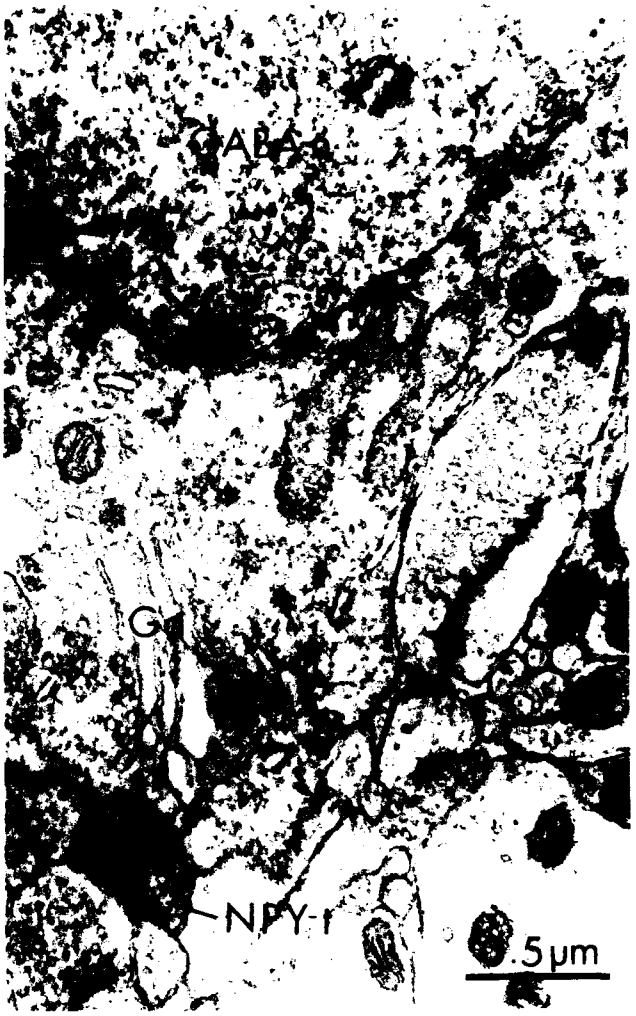

FIGURE 6. NPY-immunoreactive terminal contacting a GABAergic perikarya in the supragranular lamina of rat somatosensory cortex. The electron micrograph shows silver grains (sg) over a perikaryon indicating GABA-immunoreactivity (GABA-n over nucleus). This perikaryon is in direct contact *(arrowheads)* with an NPY-terminal (NPY-t) containing numerous small vesicles. No synaptic specialization is evident at the point of contact. Instead, saccules of the Golgi apparatus (Ga) are clustered in the vicinity.

ever, a few NPY-immunoreactive cell bodies without detectable levels of GABA-immunoreactivity were found in the cortex. In addition, dually labeled neurons were detectable in the striatum. In both regions, many more of the cell bodies in the vicinity of NPY-immunoreactive neurons were immunoreactive only for GABA.

In the cerebral cortex, the presence of three population of neurons, *i.e.*, GABA+/NPY+, GABA+/NPY− and GABA−/NPY+, was confirmed by electron microscopic

identifications of singly and dually labeled somata, dendrites and axon terminals. FIGURE 4 shows an example of a neuron exhibiting immunoreactivity for both GABA and NPY within the cell body. The somata of NPY-neurons were frequently in direct contact with the cell body of glial cells.

In the striatum, most of the somata, dendrites and axons contained either NPY or GABA. However, somata and dendrites containing immunoreactivity for NPY and GABA also were detected (FIG. 5). In contrast, dually labeled axons were not found, even though they were prevalent in the cerebral cortex in the same Vibratome sections. We also noted that the dually labeled neurons in the striatum differed markedly from most other GABAergic neurons in the vicinity in that the density of silver grains reflecting GABA-immunoreactivity was lower and that the frequency of synaptic inputs along proximal dendritic shafts also was lower. The dually immunoreactive neurons often were synaptically associated with GABAergic terminals. Thus, one possible explanation for the dually immunoreactive neurons in the striatum is that they contain GABA by uptake mechanisms, but do not synthesize or release GABA as a transmitter.

The Synaptic Relation between NPY- and GABA-Containing Neurons

In both cerebral cortex and striatum, NPY-terminals frequently form direct contacts simultaneously with dendrites and axons that, in turn, are associated with each other

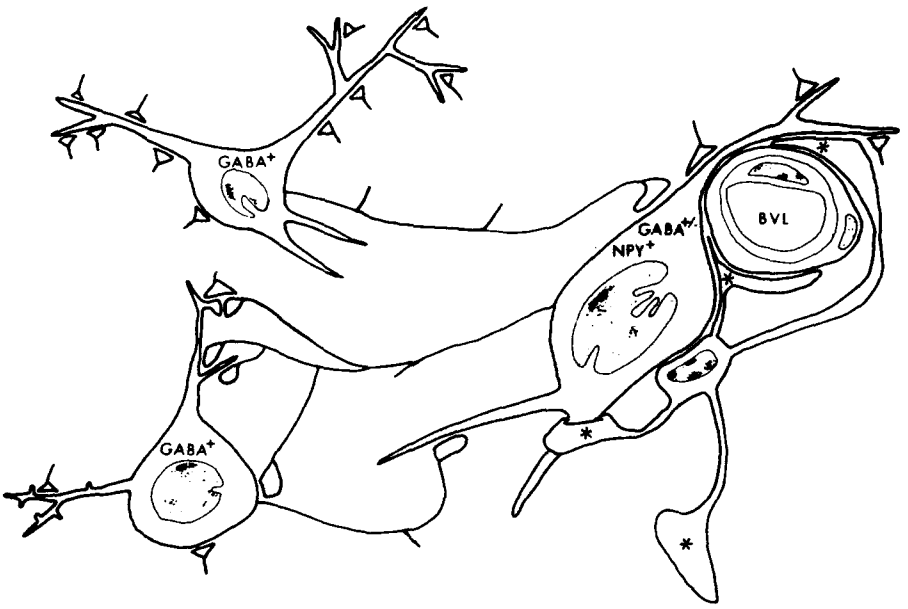

FIGURE 7. Schematic representation of cellular associations among GABAergic and NPY-containing neurons, glial cells and blood vessels in the striatum and cerebral cortex. NPY-immunoreactive neurons *(shaded grey)* innervate somata, dendrites and spines of GABAergic neurons. These NPY-receptive GABAergic neurons (GABA+, *lower left*) receive few inputs from non-NPY terminals. In contrast, many of the GABAergic neurons without apparent input from NPY-neurons (GABA+, *upper left*) show numerous contacts with non-NPY terminals *(triangles with extension)*. The NPY neurons, whether or not coexisting with GABA, are frequently adjacent to glial cells or their processes (*asterisks* within cytoplasm) or to blood vessels. BVL = blood vessel lumen.

TABLE 1. Cellular Association between NPY-ir Axon Terminals and GABA-ir Neurons in Cerebral Cortex and Caudate-Putamen Nuclei (in Percent)

	Cerebral Cortex	Caudate Putamen
Synaptic contacts	n = 179	n = 87
with somata		
GABA-ir	4	4
unlabeled	11	0
with dendrites		
GABA-ir	4	4
unlabeled	57	51
with spines		
GABA-ir	0	4
unlabeled	24	36
Juxtaposition with axons	n = 87	n = 14
en passant	86	93
junctional	14	7

through symmetric (putatively inhibitory[21]) synaptic junctions. Dual labeling for GABA and NPY has confirmed that some of the symmetric synapses contacted by NPY terminals are GABAergic.

Previous studies have also shown that NPY-neurons in the ventral striatum receive direct input from GABAergic axon terminals.[19] Conversely, we have also observed direct contacts between NPY-terminals and GABAergic somata or dendrites in the dorsal striatum and cerebral cortex (FIG. 6). The contacts between GABAergic somata and NPY-terminals rarely exhibit identifiable synaptic specializations. Instead, the two membranes merely face each other without interposition by glial processes. This, together with Colmer's earlier report that NPY failed to exert electrophysiological effects at cell bodies,[38] suggests that some of the mechanism mediating intercellular interaction with NPY may be biochemical rather than electrochemical. For example, the organized stacking of the Golgi apparatus near the site of contact may reflect NPY's modulation of peptide processing.

Although the proportion of GABAergic dendrites receiving synaptic inputs from NPY-terminals is low (8% in both the dorsal striatum and cortex), few other terminals innervate the NPY-receptive GABAergic dendrites (FIG. 7). Other GABAergic neurons in the vicinity that are without detectable NPY-input usually show more numerous (2.4:1) axo-dendritic inputs. Thus, NPY may modulate a select population of GABAergic neurons that are characterized by their relatively sparse innervation. Since fewer other synapses compete with NPY's input, NPY may exert potent influence on a specific class of GABAergic neurons.

The Role of NPY in Excitatory Transmission

Pharmacological studies using *in vitro* hippocampal slice preparation indicate that NPY may inhibit the release of excitatory transmitters by blocking Ca^{2+} influx at the terminal[38] (also reviewed in the next paper). In support of this, Hendry *et al.*[10] have shown that NPY-terminals in the monkey cerebral cortex can occur directly across asymmetric types of synaptic clefts that are considered to be the sites for excitatory neurotransmission.[21] NPY-terminals at these sites contact both the presynaptic terminals and postsynaptic dendritic spines, but do not exhibit any synaptic specializations.

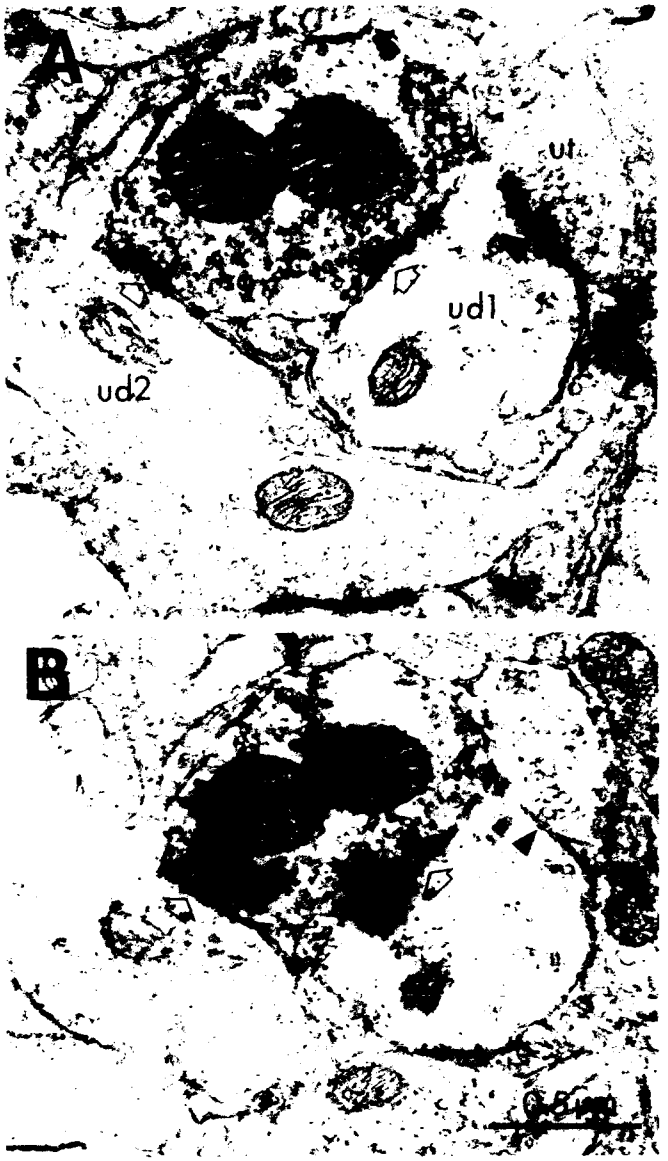

FIGURE 8. Axo-axonic relationship between NPY-GABA terminals and unlabeled terminals forming asymmetric junctions in the rat somatosensory cortex. The electron micrographs, **(A)** and **(B)**, taken from serially collected ultrathin sections, show silver grains (sg) and peroxidase reaction product indicating the coexistence of GABA with NPY (NPY/GABA-t). This terminal makes symmetric synaptic junctions *(open arrow)* with two nonimmunoreactive dendrites (ud1 and ud2). An unlabeled terminal (ut) forms an asymmetric synaptic junction to ud1 in one (*closed arrow* in A) but not the adjacent section (*arrowhead* in B). NPY/GABA-t and ut show parallel alignments of their plasma membranes at points of contact *(arrowhead pairs)*. Synaptic vesicles are clustered near the region of axo-axonic contact. However membrane specializations usually associated with synapses are not evident. (Modified from REF. 7.)

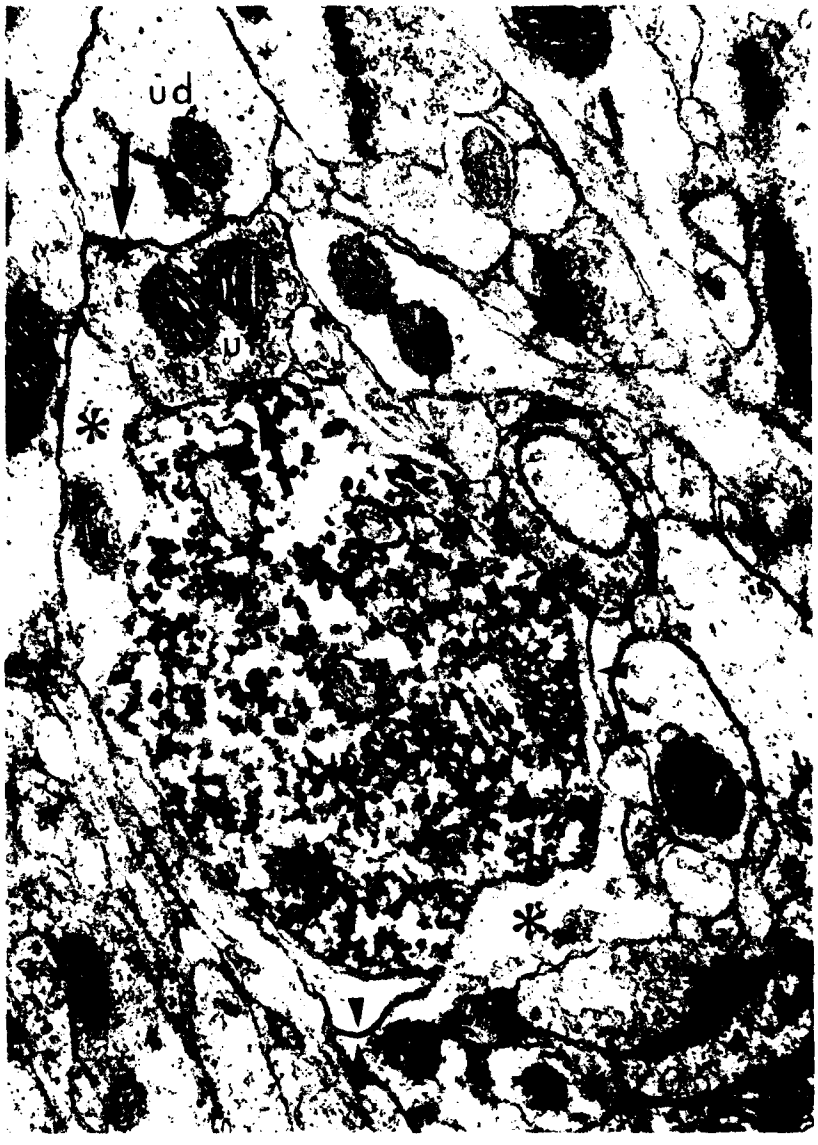

FIGURE 9. The association of an NPY-dendrite with glial processes in the dorsal striatum of rat brain. The electron micrograph shows a diffuse distribution of peroxidase reaction product indicative of NPY-immunoreactivity within a dendrite (NPY-d). NPY-d receives synaptic input from an unlabeled terminal (ut). The same axon forms symmetric junction with another unlabeled dendrite (ud). The NPY-d is surrounded by a process that exhibits irregular contours and gap-junctional association with other processes *(arrowhead pairs)*. These morphological characteristics indicate that they are glial (*asterisks* in cytoplasm). (Modified from REF. 6.)

We have compared the relative frequency of NPY-terminals forming direct contacts with synaptic junctions in the rat striatum and cortex. The results indicate that approximately half of all NPY-immunoreactive terminals in the cortex are juxtaposed to other axons (TABLE 1). Of them, 14% occur directly across synaptic junctions formed by other terminals. FIG. 8 shows an example of one such arrangement between pairs of terminals and dendrites. Axo-axonic interaction is suggested by the parallel alignment of the plasma membrane of the two terminals and the clustering of vesicles near both of the aligned membranes. Within favorable planes of section, some of the NPY-immunoreactive axons at synaptic junctions also form symmetric synaptic junctions onto a dendrite receiving input from the neighboring axon terminal. The synaptic junction formed between the neighboring unlabeled terminal and the common dendrite is asymmetric, suggesting that it is an excitatory synapse.[21]

In striatum, the relative frequency of juxtaposition between NPY-terminals and other axons was approximately 15% (TABLE 1). Of these, only 7% of the terminals formed parallel alignments of the plasma membrane simultaneously with that of the presynaptic terminal and postsynaptic dendrite. These observations suggest that more of the NPY-terminals in the cerebral cortex may be involved in the modulation of synaptic transmission. The NPY-immunoreactive terminals occurring at synaptic junctions not only form an organized association with the neighboring presynaptic terminal, but also the common dendrite. Thus, NPY may modulate non-NPY synaptic transmission by altering the release of other transmitters and also through postsynaptic mechanisms involving second messengers.[39]

NPY-Neurons and Degeneration

The NADPH-diaphorase-positive NPY neurons have been noted by some groups to be resistant to degeneration caused by NMDA receptor-specific excitotoxicity and diseases such as Huntington's chorea both in cortex and in the striatum.[36,40-43] The possibility that NADPH-diaphorase itself may play a role in protecting the neurons from excitotoxicity has been suggested.[44] Our ultrastructural studies indicate that two additional features of NPY-neurons may contribute towards their excitotoxicity-resistant phenotype. NPY somata and dendrites, whether in the cortex or striatum and whether or not coexisting with GABA show sparse distributions of synapses along the plasma membrane (see FIGS. 1, 4, 5, 7 and 9). Thus, in comparison to the frequency of synaptic inputs observed with non-NPY GABAergic neurons in the vicinity, the density of asymmetric synaptic junctions reflecting excitatory input is less than half. Therefore, the NPY-neurons may also have lower densities of glutamate receptors. In addition, dendrites and somata of NPY neurons are ensheathed by glial processes that form gap junctions with one another (FIG. 9). Thus, the high-capacity uptake sites for glutamate within glial processes[45] may serve to protect NPY-neurons from excitotoxicity caused by the release of the excitatory transmitters.

The Role of NPY in Catecholamine Turnover

One striking feature of the striatum is its rich innervation by catecholaminergic terminals. These terminals are mostly dopaminergic within the dorsal caudate-putamen nucleus. Ventrally (in n. accumbens), more of the catecholaminergic fibers contain norepinephrine.[46] Previous pharmacological studies have suggested that the release of dopamine may be modulated by NPY, since NPY can stimulate the turnover of dopamine.[3] Conversely, other studies have suggested that the NPY-containing neuronal

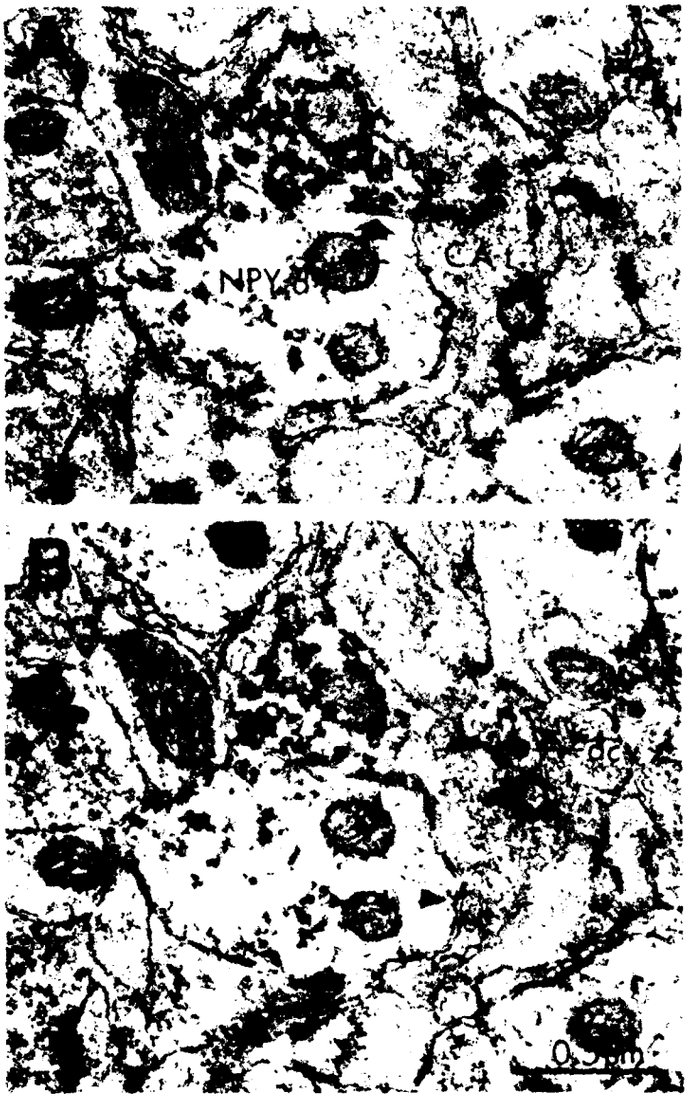

FIGURE 10. The relationship between NPY neurons and catecholaminergic axons in the ventral striatum of rat brain. The electron micrographs taken from serially collected ultrathin sections, show an NPY-terminal (NPY-t) containing numerous small clear vesicles and forming a symmetric synaptic junction with an NPY-dendrite (NPY-d) in (**A**) (*arrow*). The parallel alignment of the pre- and postsynaptic membranes is less apparent in (**B**) (*left arrowhead*). An axon containing numerous small clear vesicles and one dense-core vesicle (dcv) is catecholaminergic (CA-t), since silver grains recur over the cytoplasm in serial sections. The plasma membrane of the CA-t is in direct contact with that of NPY-d (*right arrowhead* in B) and the NPY-t. (Modified from REF. 6.)

system may be under the tonic influence of the nigrostriatal dopamine system, since 6-hydroxydopamine lesion of the nigrostriatal dopaminergic neurons leads to a marked increase in the number of NPY-immunoreactive perikarya.[47] However, since the juxtaposition of catecholaminergic fibers and NPY-neurons had not yet been demonstrated directly *in situ*, a possibility remained that the function of the two transmitters was mediated through the interposed neurons or glia.

Dual electron microscopic immunolabeling further elucidated the cellular relationship between neuronal cell bodies and processes that contain NPY or are catecholaminergic. No axo-somatic synapses were detected between catecholaminergic terminals and NPY-containing cell bodies in our analysis of striatum or cortex. However, another group using the immunoautoradiographic method to label NPY-cell bodies and catecholaminergic fibers by the immunoperoxidase method have shown direct axo-somatic synapses between the two.[48] This difference in results is likely to be attributed to the differences in sensitivity for labeling the catecholaminergic fibers.

TABLE 2. Targets of NPY-ir Axons in Striatum (in Percent)

	Caudate Putamen	N. Accumbens
Synaptic contacts	n = 190	n = 133
with somata		
NPY-ir	0	1
unlabeled	2	5
w/CA input[a]	0	0
with dendrites		
NPY-ir	0	1
unlabeled	26	25
w/CA input[a]	4	2
with spines		
NPY-ir	0	0
unlabeled	15	24
w/CA input[a]	0	0
unidentifiable	58	47

[a]*w/CA input = processes also receiving catecholaminergic input within plane of section.*

We additionally analyzed the relationship between catecholaminergic fibers and distal parts of NPY-neurons. The results indicate that catecholaminergic fibers rarely innervate NPY-dendrites. Instead, fine glial processes course between the two (FIG. 3). Occasionally (4% in dorsal striatum, 1% in ventral striatum), catecholaminergic axons converge with NPY-axons onto common dendrites. More frequently, the two types of fibers course past each other towards presumably divergent destinations. In a single case, a putatively convergent association of NPY- and catecholaminergic axon terminals onto a common dendrite was found accompanied by direct contacts between the two terminals (FIG. 10). It is intriguing to consider that these may be sites for the mutual modulation of release by the two transmitter systems, as has been suggested by pharmacologists.

SUMMARY

NPY-neurons in the striatum and cortex have many morphological and chemical features in common. They are intrinsic, medium sized, aspiny and exhibit ultrastructural

characteristics typical of neurons undergoing active synthesis and release of peptides. Most of the NPY-neurons in the two regions coexist with somatostatin, exhibit high levels of NADPH-diaphorase and are resistant to degeneration associated with Huntington's disease. Ultrastructural analysis suggests that the ensheathment by glia and sparsity of asymmetric (putatively excitatory) inputs may render NPY neurons resistant to excitotoxicity. Although NPY-neurons receive few inputs, they make numerous contacts with dendrites within a small region of the neuropil. Among their targets are GABAergic neurons. These NPY-receptive GABA neurons differ from other GABAergic neurons in the vicinity in that they receive few other inputs along their somata and proximal dendrites. This suggests that NPY may exert more influence on a specific class of GABAergic neurons. Many more of the NPY-terminals are found at sites that would be strategic for the simultaneous modulation of the release of transmitters and postsynaptic responses.

The differences among NPY-neurons in the striatum versus cerebral cortex are mainly chemical. Most notably, the NPY-neurons are GABAergic in the cortex and not GABAergic in the striatum. In addition, some of the NPY-axons in the ventral portions of striatum and cerebral cortex may be catecholaminergic, and thus originate in brainstem areas recognized to contain NPY and epinephrine or norepinephrine. NPY- and catecholaminergic fibers converge onto same dendrites. Thus, the two transmitters may interact through intercellular biochemical pathways postsynaptically. Finally, the sites where the two fibers directly contact each other may be where NPY stimulates the turnover of dopamine.

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Modulation of Synaptic Tranmission in Hippocampus by Neuropeptide Y: Presynaptic Actions[a]

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INTRODUCTION

The hippocampal formation contains a significant amount of Neuropeptide Y (NPY), expressed by neurons and contained in their fibers in the dentate gyrus and hippocampus proper.[1] The stratra radiatum and oriens of hippocampus, which represent the apical and basal dendritic fields, respectively, of the pyramidal neurons of areas CA1 and CA3, and which contain the presynaptic fibers and terminals impinging on the pyramidal cell dendrites,[2] demonstrate some of the highest concentrations of NPY binding sites anywhere in the brain, although there are few or no binding sites in the cell body layers of either CA1 or CA3.[3] While there is no precise correspondence of NPY-containing fibers and binding sites, there is also no gross mismatch, suggesting that locally-released peptide could act at nearby receptors.

NPY has been demonstrated to have both pre- and postsynaptic actions in peripheral tissues such as vas deferens and vascular smooth muscle (Wahlestedt[4] and this volume). Because of the well characterized synpatic responses in hippocampus, and the presence of this peptide and its receptors in the region, I tested the hypothesis that NPY affects synaptic transmission in the rat hippocampal since preparation, *in vitro*.

METHODS

Details of the preparation and recording apparatus have been published elsewhere.[5–8] Briefly, transverse slices (400–450 μm thick) were prepared from hippocampi taken from male Sprague-Dawley rats (100–200 g), and perfused at 1.5–2 ml min^{-1} with artificial cerebrospinal fluid (ACSF), while held submerged on a nylon mesh in a recording chamber (300 μl volume). The ACSF was saturated with 95% O_2, 5% CO_2, and held at constant temperatures (± 0.5°C) between 32 and 35°C by a water jacket. A valve permitted the rapid switching between source reservoirs for peptide and drug application via the perfusate. The experimental preparation is illustrated in FIGURE 1. Extracellular field potentials were recorded with 2 M NaCl-filled glass microelectrodes (2–10 MΩ) placed in the cell body layer of CA1, while intracellular recordings were made with 2 M K^+

[a]Support for these studies came from the Medical Research Council of Canada. The author is a Medical Scholar of the Alberta Heritage Foundation for Medical Research.

acetate-filled microelectrodes (90–180 MΩ). A bipolar stimulating electrode was placed in stratum radiatum between areas CA2 and CA1, or on other pathways as described. Square-wave monophasic pulses (50–200 μs, 2–25 V) were delivered to this electrode from a stimulus isolation unit. Stimuli were generally delivered at 0.1 Hz.

Orthodromic stimulation of stratum radiatum elicited a field potential in the cell body layer recorded by the extracellular microelectrode. The population spike (PS) component of the field potential was measured from the peak of the negative wave to the peak of the following positive one[5] (FIG. 1, inset left). Once the population response had been stable for at least 20 minutes, in many experiments a potassium acetate-filled microelectrode

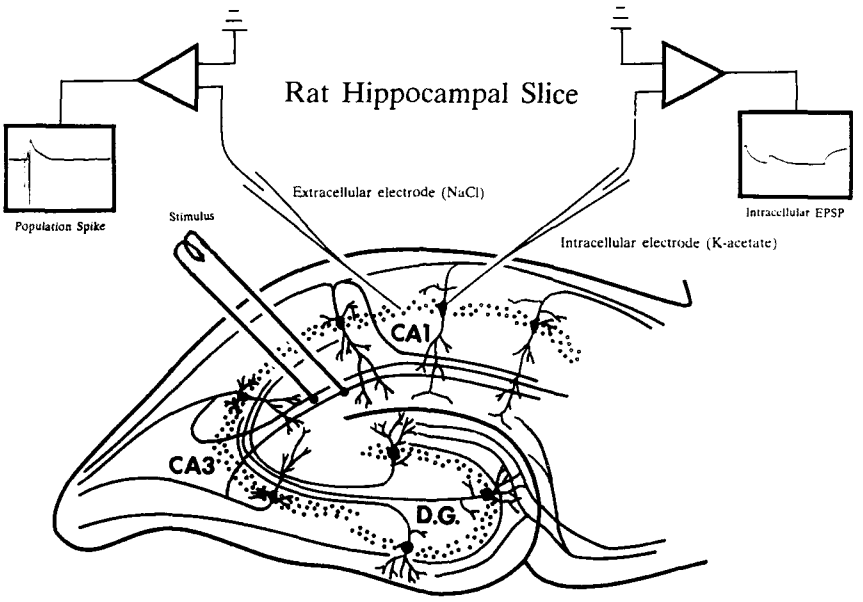

FIGURE 1. Schematic diagram of transverse rat hippocampal slice, showing synaptic relationships between principal neurons and the placement of recording and stimulating electrodes. *Inset at left* illustrates a typical extracellular field potential recording, while *inset at right* illustrates an intracellular recording of an excitatory postsynaptic potential (EPSP) evoked in a CA1 pyramidal cell by stimulation of stratum radiatum during a hyperpolarization induced by a pulse of current applied via the electrode. Abbreviations: CA1—area CA1 of hippocampus proper; CA3—area CA3 of hippocampus proper; D.G.— dentate gyrus.

was lowered into the cell body layer nearby and a CA1 pyramidal neuron was impaled and identified by standard criteria such as resting membrane potential, action potential waveform, and a response to orthodromic stimuli.[6,7] Square-wave, constant-current hyperpolarizing pulses were passed via the bridge circuit of the amplifier to prevent the neuron from reaching action potential threshold when stratum radiatum was stimulated 40 msec after the start of the pulse[6,7] (FIG. 1, inset right). Orthodromic stimuli were varied in intensity in regular steps from near threshold for the EPSP to just below action potential threshold. Once resting membrane properties and synaptic responses had stabilized, usu-

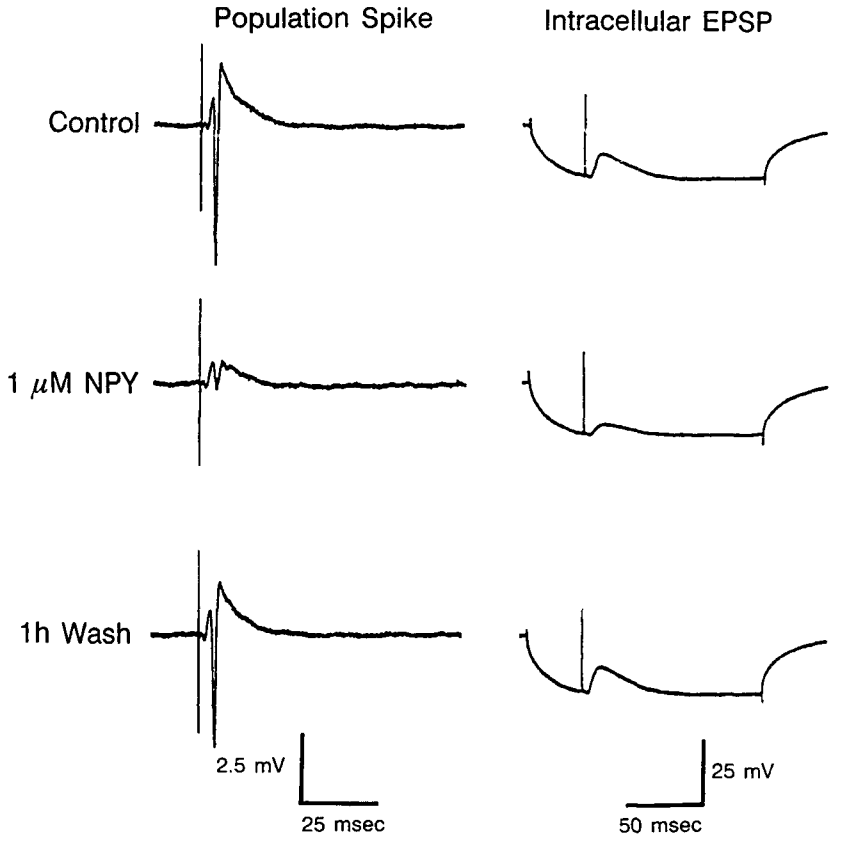

FIGURE 2. Effects of 1 μM NPY on orthodromic synaptic potentials in CA1. Simultaneous extracellular *(left)* and intracellular *(right)* recordings were made from the cell body layer and a pyramidal cell, respectively. Stratum radiatum was stimulated 40 msec after the start of a hyperpolarizing current pulse applied to intracellular electrode. NPY inhibited population spike (PS) amplitude by about 90%, and inhibited the EPSP by about 40% in this experiment. Synaptic responses recovered to near control values after about 1 hour washout with control ACSF.

ally after 20 minutes or longer, control responses were established and experiments were initiated.

Data were averaged and stored on floppy diskettes and analyzed on-line and from tape using a Nicolet 4094 digital oscilloscope. D.C. coupled chart recordings were made of resting membrane potential and injected current. Critical parts of experiments were recorded either on FM tape or digitized and recorded on videocassette. Neurons served as their own controls for statistical purposes. Student's paired *t* test was used unless otherwise noted. All data are from preparations exhibiting substantial recovery from any peptide or drug effects. Unless otherwise noted, amidated porcine sequence NPY 1–36 was used in these experiments.

RESULTS AND DISCUSSION

Action of NPY in CA1

Orthodromic stimuli elicited stable postsynaptic responses in area CA1 (FIG. 2). Bath application of NPY at 1 μM caused an inhibition of the extracellularly recorded PS by about 90% of control (FIG. 2, right). The effect of NPY was reversible upon prolonged (≥1 h) washout. The inhibition of the PS by NPY was dependent on the concentration of NPY applied; threshold for the effect was around 10 nM and the EC_{50} was about 300 nM.[5]

To test whether the ability of CA1 pyramidal cells to generate action potentials was affected by NPY, their axons were stimulated antidromically by a bipolar electrode placed in alveus, and the resulting field potential was recorded. Application of 1 μM NPY did not alter the amplitude of the antidromically-evoked field potential.[5]

Because the effect of NPY in CA1 was consistent with an inhibition of synaptic transmission at the stratum radiatum-CA1 synapse, an additional microelectrode was placed in the cell body layer and used to impale a CA1 pyramidal neuron (FIG. 1).

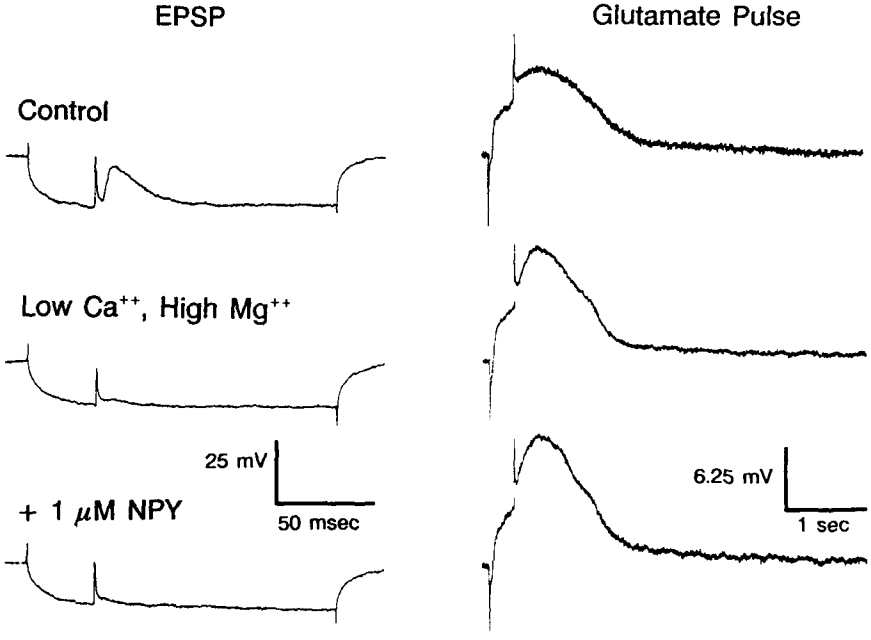

FIGURE 3. NPY does not affect glutamate responses in CA1 neurons. Digitally averaged (n = 8) responses of CA1 pyramidal neuron to stratum radiatum stimulation *(left)* or to a brief (250 msec) pulse of glutamate *(right)* applied ionophoretically from an electrode placed near the neuron's apical dendrites *(upper traces)*. Note different time and voltage scales for the two responses. The two stimuli were presented alternately to the neuron at 0.05 Hz. Synaptic transmission was interrupted by application of a bathing medium of containing 0.5 mM Ca^{++}, 3.5 mM Mg^{++}, with the other constituents being as in control ACSF, to preclude an indirect, synaptically mediated effect of glutamate on the neuron *(middle traces)*. Application of 1 μM NPY *(lower traces)* did not inhibit response of neuron to glutamate. (From Colmers *et al.*[6] Reprinted by permission from the Physiological Society.)

Stimulation of stratum radiatum while passing a hyperpolarizing current pulse into the cell elicited a fast EPSP. Bath application of 1 μM NPY resulted in an inhibition of the EPSP amplitude by about 40% of control, which reversed upon prolonged washout (FIG. 2, left). The time course of NPY-mediated inhibition and recovery subsequent to washout was the same for the EPSP and the PS.[6,7]

Extensive experiments were performed to determine whether NPY affected any active or passive membrane properties of the postsynaptic CA1 neurons.[6] 1 μM NPY had no effect on the resting membrane potential of CA1 neurons. The input resistance, estimated from the voltage response of the neuron to families of hyperpolarizing and depolarizing current pulses, was also not affected by NPY. NPY also had no effects on the amplitude, duration, threshold, or number of action potentials evoked by a depolarizing current pulse in CA1 cells.[6] Therefore, there was no direct action of NPY on the postsynaptic properties of CA1 cells measured at the cell soma.

There remained three possible explanations for the effects of NPY on synaptic transmission. 1) NPY could cause a very localized reduction in the input resistance of the dendrites, which, because of the great electronic length of the apical dendrites, might not be observed while recording in the soma, but could have a profound effect on the amplitude of the EPSP. 2) NPY could be acting as an antagonist at the postsynaptic receptors for the excitatory transmitter, glutamate. 3) NPY could inhibit synaptic transmission by a presynaptic action. To test these hypotheses, pulses of glutamate were applied iontophoretically from a pipette placed next to the apical dendrites of impaled CA1 neurons. If NPY were acting via either mechanisms 1) or 2), the response to glutamate should be inhibited by NPY application to a similar degree as the EPSP. This was tested in the absence of synaptic transmission, using a low Ca^{++}, high Mg^{++} solution to preclude indirect excitation of other presynaptic elements by glutamate application.[6] However, application of 1 μM NPY caused no change in the amplitude of the response of these neurons to glutamate (FIG. 3).

Site and Mechanism of Action in CA1

The above series of experiments led to the conclusion that NPY was inhibiting synaptic transmission by an action at a presynaptic site, as there was no evidence for any, even subtle, effects of the peptide on the postsynaptic neuron. The next series of experiments were designed to identify the presynaptic site of action of NPY, and to attempt to elucidate the mechanism by which it acts.

The hippocampal circuitry is shown in schematic form in FIGURE 4. As indicated, the majority of synaptic input to CA1 arises from the Shaffer collaterals, the ipsilateral branches of CA3 pyramidal cell axons.[2] Experiments showed that NPY had no effects on the active or passive membrane properties of CA3 pyramidal neurons.[7] Because the peptide could affect the excitability of the Shaffer collateral axons or their terminals,[8] a stimulating electrode was placed in stratum radiatum of CA1 (stimulus site 2, FIG. 4), and antidromically-evoked field potentials were recorded with extracellular microelectrodes in the cell body layer of area CA3. However, NPY had no effect on the amplitude of the antidromic field potentials in CA3, indicating that there was no effect on the excitability of the axons or terminals in CA1.[7] In other experiments, the amplitude of the afferent volley (a component of the field potential recorded in stratum radiatum of CA1 in response to orthodromic stimuli), which represents the number of presynaptic axons discharging in response to the stimulus,[10] was not affected by NPY. Thus, two lines of evidence were consistent with there not being any effect of the peptide on the excitability of the presynaptic axons.

The possibility remained that NPY was acting at the presynaptic terminals themselves

to inhibit release of neurotransmitter, by affecting conductances which are not active at rest, and would therefore not greatly affect the excitability of the terminals and axons as tested above. Two possible mechanisms for this are: 1) the activation of a voltage-dependent potassium conductance in the terminals, hyperpolarizing it and making depolarization less likely upon stimulation,[11] and 2) the inhibition of voltage-dependent calcium influx, upon which transmitter release depends.[12] It is known that presynaptic terminals in rat CNS possess potassium channels which are very sensitive to the action of 4-aminopyridine (4-AP).[13] To test possibility 1), NPY's effects on synaptic transmission were examined in the presence of low concentrations of 4-AP.

Application of 10 μM 4-AP, a concentration known to markedly inhibit potassium channels in presynaptic terminals,[13] but which has little effect on the transient, voltage-dependent "A" potassium current in the soma,[14] caused a marked increase in transmitter

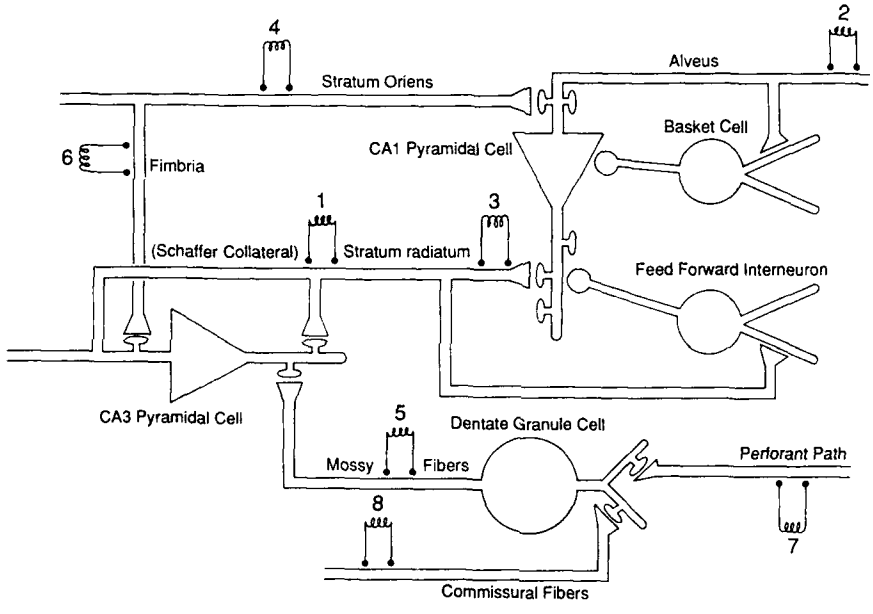

FIGURE 4. Schematic diagram of synaptic circuitry in hippocampus, illustrating stimulating electrode placement for experiments. See text for details.

release to a given stimulus (FIG. 5). Application of 1 μM NPY in the presence of 10 μM 4-AP had no effect on synaptic transmission (FIG. 5). Thus, it at first appeared that NPY's effect was via an activation of a 4-AP sensitive K^+ conductance.

However, there was another interpretation for the results. By blocking voltage-dependent K^+ channels in the presynaptic terminal, the repolarization of the membrane at the terminal was delayed. If NPY were inhibiting the activation of only a portion of the voltage-dependent Ca^{++} channels in the presynaptic terminal, then the delayed repolarization induced by 4-AP could allow enough calcium to enter the terminal to cause transmitter release, overwhelming the normal inhibition caused by NPY (FIG. 6). The extracellular Ca^{++} was therefore reduced, in the presence of 4-AP, to a concentration which restored the pre-4AP levels of synaptic transmission. This presumably meant that the amount of Ca^{++} entering the presynaptic terminals was about the same as before

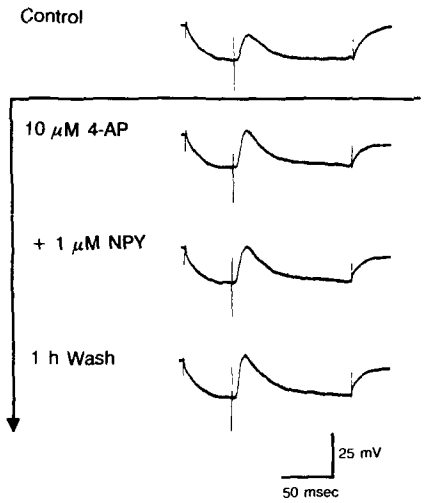

FIGURE 5. Effect of 4-aminopyridine (4-AP) on NPY inhibition of stratum radiatum EPSP recorded intracellularly in a CA1 pyramidal neuron. 4-AP increased synaptic transmission in response to identical stimulus as control. Application of 1 μM NPY in the presence of 4-AP did not inhibit EPSP.

4-AP was applied. The concentration of extracellular Ca^{++} needed to restore the pre-4-AP levels of transmitter release was found empirically to be 0.70 mM. Under these conditions, application of 1 μM NPY once again inhibited synaptic transmission[7](FIG. 7).

Further experiments were conducted using a higher concentration of 4-AP, 50 μM. As in the earlier experiments, NPY was ineffective in the presence of the higher concentration of 4-AP. When the Ca^{++} concentration was lowered to 0.70 mM, NPY was still ineffective. However, lowering extracellular Ca^{++} to 0.50 mM restored NPY's ability to inhibit synaptic transmission.[7]

The results are therefore not consistent with the presynaptic inhibition by NPY resulting from its activation of a 4-AP-sensitive K^+ conductance. It is furthermore unlikely that NPY activates another, 4-AP insensitive, K^+ conductance. This is because the input resistance of the presynaptic terminal would be increased due to the blockade of a K^+ conductance by 4-AP. If NPY were to activate a 4-AP insensitive K^+ conductance, the hyperpolarization, and therefore presumably the action of NPY thus caused, would be *greater* in the presence of 4-AP than in control.

However, the data are consistent with the NPY's presynaptic action resulting from the inhibition of a portion of the Ca^{++} influx into the presynaptic terminal. In support of this, experiments on rat dorsal root ganglion (DRG) neurons in culture[15] have demonstrated that NPY has a very potent and prolonged inhibitory effect on calcium currents recorded in isolation.

Coupling of the NPY Receptor to Effectors

Experiments on DRG cells in culture indicated that NPY receptors inhibited the opening of voltage-dependent calcium channels via the activation of a GTP-binding protein which was sensitive to the action of the bacterial exotoxin produced by *Bordetella pertussis*, pertussis toxin (PTX). This toxin acts by entering the cell and irreversibly placing an ADP-ribose molecule onto the GTP binding site of some GTP binding proteins (G-protein), which irreversibly inactivates them, thus decoupling the receptor from its

effector by eliminating the intermediary.[15,16] If NPY acts at the presynaptic terminal by decreasing calcium influx, then this action might be sensitive to the action of PTX. The hypothesis was therefore tested whether the action of NPY on synaptic transmission was sensitive to the actions of PTX. Rats were injected with 1.5 μg PTX into the lateral cerebral ventricle, and allowed to survive for 3 days, for the toxin to act.[8,17] Slices were prepared from the hippocampi of these animals in an otherwise identical manner as before.

Physiological properties of slices prepared from PTX pretreated animals did not differ significantly from those prepared from control animals. Intracellular recordings of CA1 neurons in slices from PTX pretreated animals demonstrated no significant difference from those of untreated animals in their resting membrane properties or action potential parameters. Stimulation of stratum radiatum in these slices evoked an intracellularly recorded EPSP and an extracellularly recorded PS. Application of 1 μM NPY to slices from PTX pretreated animals inhibited excitatory synaptic transmission: The EPSP was

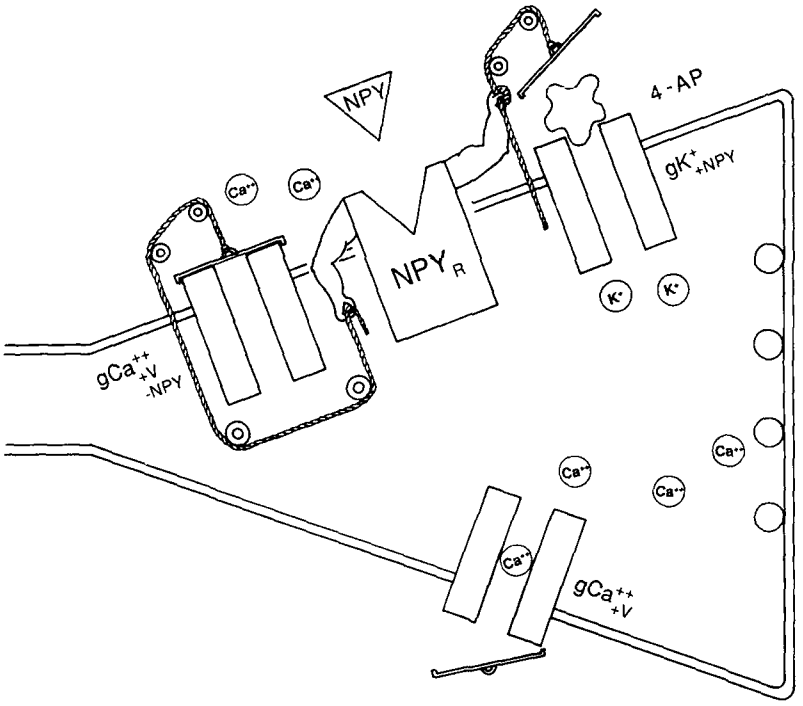

FIGURE 6. Cartoon illustrating possible interpretations for the 4-AP mediated blockade of NPY inhibition of transmitter release at a presynaptic terminal. The NPY receptor (NPY_R) could be coupled so as to activate a 4-AP sensitive potassium channel ($gK^+ + NPY$) or to inhibit activation of voltage-dependent calcium channels ($gCa^{++} + V$, -NPY) in the presynaptic terminal. However, if NPY inhibits only a portion of the calcium channels in the presynaptic terminal, and other, NPY-*insensitive* voltage-dependent calcium channels ($gCa^{++} + V$), are present, the prolonged depolarization caused by 4-AP blockade of potassium channels could permit enough calcium to enter the terminal through the NPY-insensitive channels to permit transmitter release, thus seeming to overwhelm NPY's inhibition of the other calcium channels.

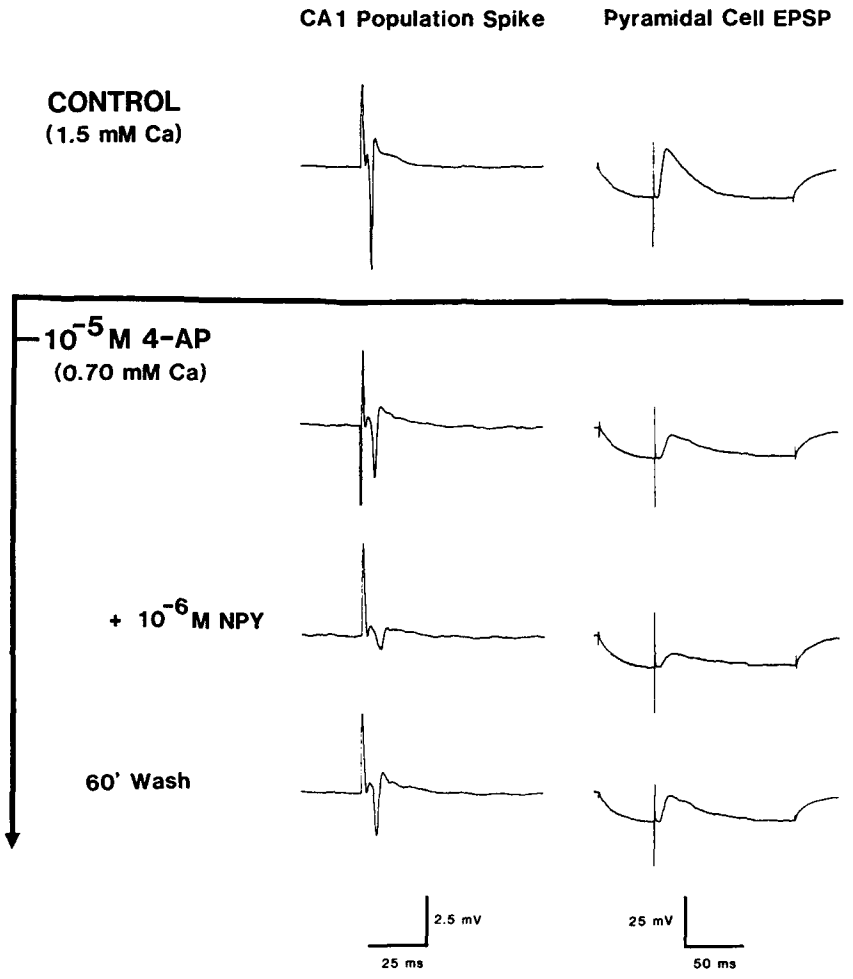

FIGURE 7. Reducing extracellular Ca^{++} concentration in the presence of 4-AP restores NPY's inhibition of synaptic transmission. Simultaneous extracellular *(left traces)* and intracellular *(right traces)* recordings in CA1 in control *(upper traces)*, and after bathing medium had been changed to one containing 10 μM 4-AP and having only 0.7 mM Ca^{++} *(lower three traces)*. Application of 1 μM NPY under these conditions inhibited synaptic responses, which recovered upon prolonged washout.

inhibited by about 40%, and the PS was inhibited by about 80%, in slices both from PTX pretreated and control animals. No significant difference between the actions of NPY was observed in the PTX pretreated group[8].

Because these results raised the possibility that PTX pretreatment had been ineffective in ADP-ribosylating the G-proteins in the hippocampus, we applied either 5-HT or baclofen, both of which elicit a hyperpolarizing response brought about by an increase in potassium conductance, mediated by a PTX-sensitive G-protein.[18] However, neither 5-HT nor baclofen had any effects on the membrane potential of the CA1 pyramidal cells,

even at very high concentrations, indicating that the PTX had been effective at eliminating G-proteins in the cell bodies of hippocampal neurons.[8] Assuming that the toxin has equal access to the presynaptic terminals, this indicates that the action of NPY at the terminal is mediated by a PTX-insensitive G-protein. Interestingly, in this study it was also observed that the presynaptic inhibitory action of baclofen on excitatory synaptic transmission in hippocampus was not affected by PTX pretreatment, although its postsynaptic effects were eliminated.[8] There may thus be a similar mechanism coupling both presynaptic NPY and $GABA_B$ receptors to the inhibition of transmitter release in hippocampus.[8,17]

Other Sites of NPY Action in Hippocampus

Excitatory Synaptic Transmission

Similar experiments have demonstrated that 1 μM NPY potently and reversibly inhibits synaptic transmission at other sites in the hippocampus. The EPSP evoked by stimulation of stratum oriens in CA1 (Site 4, FIG. 4) is inhibited by NPY by about 45% of control. In CA3 pyramidal neurons, the EPSP evoked by stimulation of the mossy fiber input pathway (Site 5, FIG. 4) is inhibited by NPY by about 45% of control. The EPSP evoked in CA3 cells by stratum radiatum (Site 1, FIG. 4) which activates the CA3-CA3 excitatory interconnections,[19] was also reduced by NPY to about 50% of control. Stimulation of the fimbria in area CA3 (Site 6, FIG. 4) evoked an EPSP which was inhibited by about 45% by NPY. By contrast, the EPSP's evoked in dentate granule cells by stimulation of either perforant path input (Site 7, FIG. 4) or commissural input (Site 8, FIG. 4) was *not* affected by NPY, although other known agents having presynaptic action, such as baclofen, muscarine and adenosine all inhibited the intracellularly recorded EPSP and extracellularly recorded PS in granule cells.[20] This correlates with the binding data, which indicate that: 1) there are no NPY binding sites in the outer two-thirds of the molecular layer, which is where the perforant path fibers terminate on the dendrites of the granule cells,[3] and 2) that the binding site density is much lower in dentate than in the hippocampus proper.[3] Because the greatest density of NPY innervation in the hippocampal formation is in the outer two-thirds of the molecular layer,[1,25] it raises the intriguing question of what actions the local release of NPY might have in this apparently insensitive region.

Inhibitory Synaptic Transmission

The action of NPY was studied on inhibitory synaptic potentials evoked in area CA1. Stimulation of stratum radiatum elicits a compound synaptic potential in CA1 pyramidal neurons, of which the EPSP is the earliest component. Following the EPSP, a fast, $GABA_A$ receptor-mediated, chloride-dependent inhibitory postsynaptic potential (IPSP) occurs.[21] This potential originates with the basket cells, inhibitory interneurons which are driven by axon collaterals of the pyramidal cells of CA1 (FIGURE 4), and which innervate the soma and initial segment of the CA1 neuron. This IPSP is generally referred to as "recurrent," since it feeds back onto the pyramidal cells, and can be evoked in isolation by stimulation of the CA1 cell axons in alveus[21] (FIG. 4). A later, slower inhibitory potential is also seen, especially at higher stimulus intensities.[21] This is mediated by a $GABA_B$ receptor, which activates a potassium conductance via a GTP binding protein, and is largely localized at the dendrites of the pyramidal cells.[18,21] This IPSP cannot be activated by alvear stimulation, and is therefore referred to as a "feed-forward" IPSP.[21]

Both feed-forward and recurrent IPSP's were elicited in single CA1 pyramidal neurons by alternate stimulation of stratum radiatum and alveus. Application of 1 μM NPY reduced the amplitude of the ISPS's evoked by stimulation of stratum radiatum to about 60% of control, the same amount as the EPSP was reduced in these neurons. However, the recurrent IPSP evoked by stimulation of the alveus was not affected by NPY at the same time as the feed-forward ISPS's were inhibited.[22]

The simplest explanation of these results is that NPY inhibits the feed-forward excitatory drive from stratum radiatum fibers, both to the CA1 pyramidal neurons, thus *indirectly* reducing the drive to the inhibitory basket cells, and *directly* reducing the synaptic excitation of the feed-forward inhibitory interneurons. However, NPY does not appear to affect the synaptic excitation of the basket cells directly, as the ISPS evoked from the alveus is not affected by NPY.

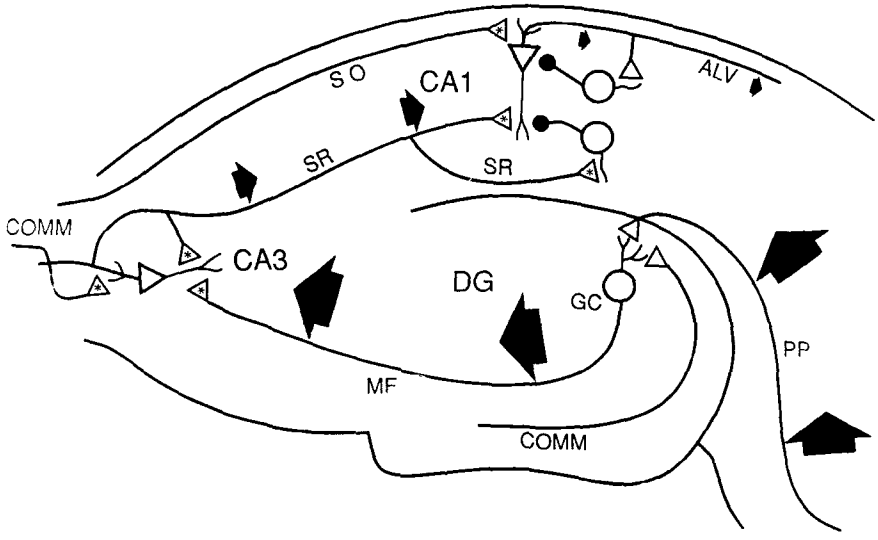

FIGURE 8. Schematic diagram of synaptic connections in hippocampus, illustrating possible action of NPY in modulating synaptic transmission in hippocampus. *Arrows* symbolize the amplitude of an excitatory input to hippocampus. As excitatory input passes along the trisynaptic pathway, NPY action would attenuate it, at inputs to both area CA3 and area CA1. *Asterisks* (*) indicate excitatory synapses inhibited by NPY. Abbreviations: ALV = alveus; COMM—commissural inputs to dentate granule cells or CA3 pyramidal cells (different pathways); GC—dentate granule cells; MF—mossy fibers; PP—perforant path; SO—stratum oriens; SR—stratum radiatum.

SUMMARY

NPY is a potent inhibitor of excitatory synaptic transmission in the rat hippocampus *in vitro*. NPY has no demonstrable actions on the active or passive properties of dendrites, cell bodies and axons of principal neurons in this area of the brain. It does appear to inhibit the release of the excitatory neurotransmitter, which is probably glutamate, from presynaptic nerve terminals. Although the mechanism of its action at the presynaptic nerve terminal is not absolutely certain, results from the 4-AP experiments are consistent with NPY inhibiting a portion of the voltage-dependent calcium channels in the terminal. This

would also be in agreement with the results obtained by others from mammalian neurons in culture, in which *NPY is a potent inhibitor of voltage-dependent* Ca^{++} channels.[15] It appears that, although the NPY receptor is most likely a G-protein linked receptor,[23] and can couple to both G_i and G_o,[23] it does not do so at the presynaptic nerve terminal in hippocampus. The NPY receptor in hippocampus has a pharmacological profile similar to the peripheral Y_2 subtype (Klapstein *et al.*, this volume). Although the Y_2 receptor has been shown to inhibit adenylate cyclase, this does not appear to be the mechanism of its action in presynaptic terminals, since the elevation of cAMP with membrane soluble analogues did not interfere with NPY's inhibitory effects (Klapstein *et al.*, this volume). However, because there is evidence: 1) that inhibition of calcium influx can occur through PTX-insensitive G-proteins,[24] evidently activated by the same receptor which also couples to a PTX-sensitive G-protein,[17] and 2) that secretory processes can be regulated directly via a G-protein dependent process, independent of the actions on Ca^{++} influx,[25] there are several further mechanisms by which NPY might potentially regulate the release of neurotransmitter at the nerve terminal.

The picture emerges from these data that the release of NPY in hippocampus would be accompanied by a reduction in the amplitude of an excitatory signal which enters the hippocampus proper. NPY effectively reduces the input amplitude of both the feed-forward excitatory and inhibitory synaptic components, without changing the properties of the postsynaptic circuitry, such as recurrent inhibition. NPY thus does not affect the input-output properties of the postsynaptic circuitry, unlike other substances known to act at inhibitory presynaptic inhibitory receptors in hippocampus, all of which have postsynaptic actions as well. Because of this, NPY's actions in hippocampus can be likened to the volume control on a good hi-fi amplifier, attenuating the input without affecting the fidelity of the amplification. The attenuation of a signal originating from entorhinal cortex would presumably increase at every stage where NPY release is elicited (FIG. 8). The actions of NPY could however be construed as being consistent with the role of an "endogenous anticonvulsant." Given the recent demonstration of a rat model of epilepsy in which the NPY/somatostatin containing interneurons of the dentate gyrus are selectively ablated,[26] and the reported selective loss of these interneurons in dentate gyrus of human patients suffering from cryptogenic temporal lobe epilepsy,[27] it is certainly conceivable that NPY plays a role in the prevention of hyperexcitability in the hippocampus.

Because there is as yet no reliable antagonist for NPY receptors, the actual physiological function it subserves in hippocampus is still a matter of speculation. However, the dramatic and potent action of NPY in the pharmacological experiments outlined above, and the very high concentration of NPY receptors in hippocampus indicate a role for this peptide in the normal physiology of the rat hippocampus. It is known that NPY/somatostatin-containing interneurons are innervated by axon collaterals of principal neurons in hippocampus, and can therefore presumably release the peptide in response to appropriate levels of stimulation. In the absence of a specific NPY antagonist, it may be possible to demonstrate appropriate release by displacement of radiolabelled NPY, as has recently been done for dynorphin release from mossy fibers.[28]

Much further work is needed on NPY's action in the hippocampus, from elucidating the exact mechanism of action of NPY at the presynaptic nerve terminal to determining under which physiologically appropriate circumstances the peptide is released. Ultimately, of course, the question to be answered is that of the physiological role of NPY in the brain of an intact animal.

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Neuropeptide Y-Cholinergic Interactions in Neocortical Tissue

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid peptide isolated from porcine brain by Tatemoto *et al.* in 1982.[1] It is widely distributed throughout the central and peripheral nervous systems where it appears to be a neuromodulator and transmitter.[2-4] The significance of NPY in regulating a variety of brain-functions is well documented, since its discovery only 8 years ago. Intracerebroventricular administration of NPY to rats results in hypotension, bradycardia and bradyapnea.[5] Injections of NPY into the hypothalamus evoke a 10-fold increase in food intake,[6] stimulate drinking[7] and inhibit LH secretion.[8] Of particular interest recently has been the potential influence of NPY-transmission in memory and cognition, which appears to be mediated in part by NPY in the cerebral cortex and hippocampus. NPY administration into the hippocampus was shown to improve learning and memory processes in the rat,[9] indicating a potential role for NPY in these higher cognitive processes. Further, Alzheimer's disease can but does not always decrease the levels of this peptide in the cerebral cortex (Ref. #10 vs 11,12).

The inconsistent effects of Alzheimer's disease on neocortical NPY levels have been particularly interesting to us, since NPY is usually co-localized with somatostatin, the levels of which are more reproducibly decreased by the disease. It therefore appears that this disease may be capable of selectively reducing the levels of one peptide without affecting the other co-localized peptide. This differential sensitivity to Alzheimer's disease may be important in understanding how it renders the somatostatin-system and potentially others hypofunctional. While considerable work has been performed on the regulation of somatostatin gene expression and turnover, less is known about the corresponding regulation of the NPY gene, especially in the neocortex. We therefore began to investigate the factors that may regulate the expression and turnover of NPY and its encoding mRNA in the cerebral cortex.

Neocortical NPY containing neurons are local circuit, intrinsic neurons.[13] They are of small to intermediate size (5–15 μm in diameter) and are characterized by their multipolar, bipolar or stellate shapes. NPY containing neuronal cell bodies are found in all layers of the cerebral cortex but appear to concentrate in layers II, III, V and VI. NPY is extensively co-localized with somatostatin within the cerebral cortex.[14] It is estimated that 80% of the NPY containing neurons also contain somatostatin and together account for approximately 2% of cortical neurons. Many NPY containing neurons also contain GABA as well as the NADPH diaphorase reaction.[15] Within the cortex, NPY axons terminate on small peripheral dendrites of pyramidal neurons (glutamatergic or aspartatergic) which in

turn project to deeper structures such as the striatum, thalamus, brain stem and other cortical regions.[16] Neuropeptide Y fibers in laminae II–VI mainly run vertically whereas fibers in lamina I generally run parallel to the pial surface.[17] In the neocortex, neuropeptide Y fibers are visualized in close proximity to blood vessels, which may constitute a morphological basis for its vasoconstrictive actions.

While many of these synaptic outputs of neocortical NPY containing neurons have been characterized, very little is known about the afferents synapsing on these NPY neurons. One likely candidate for doing so is the cholinergic system, based on several functional studies. Long-term neocortical cholinergic hypofunction in the cerebral cortex following nucleus basalis lesions elevates NPY levels by over 100% in the parietal cortex by 10 months postlesioning.[18] Even short-term changes in cholinergic transmission, such as those induced by peripheral oxotremorine injections, can significantly alter the levels of mRNA encoding for NPY in the rat parietal cortex.[19] However, while these results suggest that changes in cholinergic transmission can modulate the synthesis and levels of NPY in the cortex, they do not demonstrate direct interactions between these transmitter systems.

Cholinergic innervation in the rat neocortex consists of at least two systems, one intrinsic and one extrinsic. Intrinsic cholinergic neurons are local circuit interneurons that are found throughout the cerebral cortex. Extrinsic cholinergic input into the cerebral cortex originates from neuronal cell bodies located within the nucleus basalis magnocellularis (nBM) that project to the cerebral cortex. It is these extrinsic neurons which are among the most consistently rendered dysfunctional during the early stages of Alzheimer's disease[20,21] and are implicated in the memory and cognitive disorders associated with the disease.[22,23] However, it is conceivable that both types of cholinergic neurons modulate NPY neurons involved in memory and cognition.

In order to study the effects of cholinergic transmission on NPY expression more directly, we developed a primary neuronal cell co-culture system containing both neocortical NPY neurons and basal forebrain cholinergic neurons. Previous work from our laboratory showed that there was little or no cholinergic activity in primary cultures of the neocortex alone under these conditions, perhaps because the intrinsic cholinergic neurons did not survive the plating procedure (E. Meyer, unpublished observation). Therefore, these co-cultures should be useful in dissociating the effects of basal forebrain cholinergic neurons from the combined effects of both types of neurons (intrinsic and extrinsic) that are observed *in vivo*. This report describes our study of whether these co-cultures synthesized, stored and released NPY in a manner sensitive to changes in muscarinic receptor activity.

METHODS

Neuronal Cell Co-cultures

Primary neuron-enriched cell cultures were prepared from the cerebral cortex and basal forebrain of 1-day-old rats, essentially as described elsewhere.[24] Briefly, the basal forebrain and neocortex were isolated, digested with trypsin and mechanically dissociated. 15×10^6 cells were plated onto poly-1-lysine coated culture plates (100 mm) and maintained on Dulbecco's Modified Eagle's Medium (DMEM) containing 10% plasma derived horse serum. Three days later, cells were treated with media containing the antimitotic agent, cytosine arabinoside, which prevents the rapidly growing glial cells from proliferating. This treatment destroys approximately 90% of the glia. After a two-day period, this medium was replaced with fresh medium and the cultures were allowed

to mature for 10 to 15 days before use. The neuronal cultures contained greater than 85% neurons.

Experimental Paradigm

NPY release: All experiments were performed on 15-day-old neuronal co-cultures. Neuronal cells were washed twice with Krebs Ringer buffer, 37°C, pH 7.4, to remove the media and 5 mls of Krebs Ringer buffer, 37°C, pH 7.4, was immediately added to each plate. The various drug treatments were added and incubated with the cells for 10 min. At the end of the 10-min incubation period, NPY was measured in the Krebs Ringer buffer and cells by radioimmunoassay as described previously.[25] The incubation medium was added to ice-cold glacial acetic acid to form a final concentration of 2.0 N glacial acetic acid. The cells were scraped from each plate with 2.0 N glacial acetic acid. Total NPY levels (cellular content + KRB content) were expressed as pg/plate.

Pre-pro NPY mRNA: All experiments were performed on 15-day-old neuronal co-cultures. The various treatments were added to the medium for a time interval of 4 hrs. At the end of the incubation period, the medium was discarded and the plates rinsed with ice-cold phosphate buffered saline. The cells were then scrapped from the plates with 4.0 M guanidinium isothiocyanate buffer pH 7.0 and processed for total RNA extraction.

Pre-Pro NPY mRNA Assay

Total cellular RNA was extracted from neuronal co-cultures by the acid guanidinium-phenol-chloroform method as described by Chomczynski and Sacchi (1987).[26] Approximately 25–40 μg RNA was isolated from each 100-mm plate. The concentration of RNA isolated was quantitated spectrophotometrically by absorption at 260 nm and the purity assessed by A_{260}:A_{280} ratios. Only ratios greater that 1.9 were used for further analysis.

Pre-pro NPY mRNA was measured by dot blot analysis utilizing a rat pre-pro NPY cDNA probe that was generously provided by Dr. Janet Allen.[27] Three concentrations of total RNA from each sample were filtered under slight vacuum through Nitroplus 2000 nitrocellulose membrane (MSI, Inc., Westboro, MA) held in a Hybri-Dot manifold (Bethesda Research Laboratories). The membrane was baked in a vacuum oven for 2 hrs at 80°C. Blots were prehybridized for at least 3 hrs at 42°C and then hybridized for 16–24 hrs at 42°C using the same hybridization conditions as those used previously for Northern blots.[19] The rat pre-pro NPY cDNA was labeled by nick translation and the hybridization buffer contained 10^6 cpm/ml of [alpha ^{32}P]dCTP labeled pre-pro NPY cDNA. Dot blots were quantitated by computer image analysis with the Betascope Blot Analyzer, Model 603 (Betagen Corp., Waltham, MA). The data were calculated as the counts of ^{32}PdCTP-NPY/μg RNA that were collected over a 16-hr time period.

RESULTS

Atropine (1 μM) caused an approximately 2-fold elevation in pre-pro NPY mRNA levels in primary neuronal co-cultures after a 4-hr incubation (FIG. 1). Carbachol had no effect of pre-pro NPY mRNA in whole brain neuronal cell cultures after a 4-hr incubation period (data not shown). Total NPY levels representing the amount of NPY within the cells in addition to the amount of NPY released into the KRB (in 10 min) did not differ from control after a 4-hr incubation with atropine (1 μM) (FIG. 1). Cells were then

incubated with atropine (1 μM) for 4 hrs, fed with fresh media for 2 hrs and then assayed for total NPY levels, to determine if this time interval postatropine treatment would allow increased synthesis of NPY. Total NPY levels after this treatment also did not differ from controls (FIG. 1). These data indicate that although atropine causes a significant increase in pre-pro NPY mRNA, this increase does not appear to increase the amount of peptide within the cell.

Cholinergic muscarinic receptors are known to mediate their actions through adenylate cyclase or phosphtidylinositol metabolism. Furthermore, it was demonstrated that the NPY gene can be regulated through the cAMP second messenger system in PC12 cells.[28] Therefore, we studied the effects of atropine and forskolin, an activator of adenylate cyclase on primary neuronal co-cultures. Atropine (1 μM) and forskolin (10 μM) significantly elevated messenger RNA levels encoding NPY after a 4-hr incubation period by approximately 55% and 42%, respectively (FIG. 2). Combined treatment of these co-cultures with atropine plus forskolin elevated NPY levels by approximately 40% (FIG. 2).

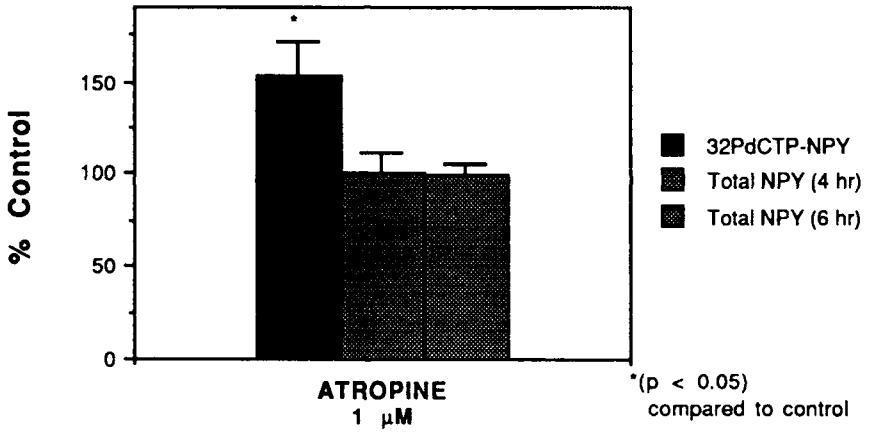

FIGURE 1. The effects of atropine on pre-pro NPY mRNA levels and total NPY levels (cellular + Krebs Ringer Buffer medium) in primary neuronal cell co-cultures (neocortex and basal forebrain) (Student *t* test).

This suggests that both of these agents may be acting through the same mechanism, *i.e.*, increasing cAMP levels.

There was no increase in NPY release over basal levels after a 10-min incubation period with the cholinergic agonist, carbachol or the muscarinic antagonist, atropine (FIG. 3). In order to determine that NPY could be released from co-cultures, cells were treated with 50 mM potassium chloride in the presence and absence of calcium. The results indicated that NPY was released from neuronal co-cultures upon potassium depolarization in a calcium dependent manner (FIG. 3).

DISCUSSION

The primary co-cultures utilized in these experiments were prepared from selective brain regions (neocortex and basal forebrain) and were designed to address neocortical

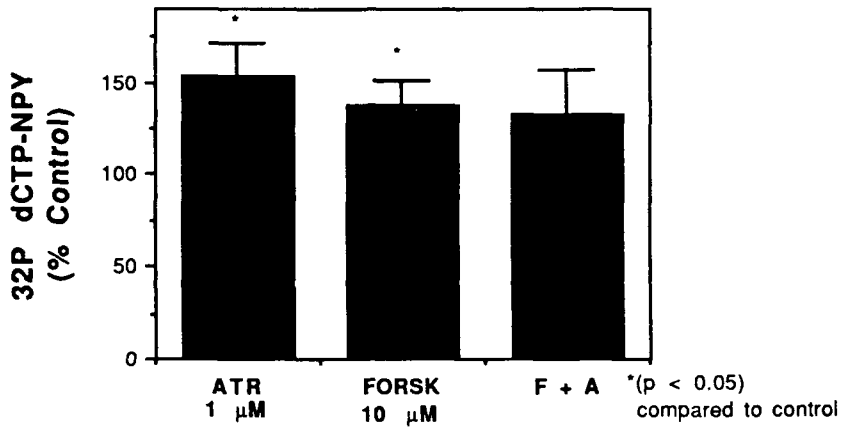

FIGURE 2. The effects of atropine (ATR and A) and forskolin (FORSK and F) on pre-pro NPY mRNA levels after a 4-hr incubation period with primary neuronal cell co-cultures (neocortex and basal forebrain) (Student *t* test).

NPY-cholinergic interactions more directly. These data indicate that this primary neuronal co-culture system can serve as a model for studying how cholinergic and other receptors regulate neuronal NPY expression in the cerebral cortex.

NPY was synthesized and released by depolarization from primary neuronal co-cultures derived from the basal forebrain and neocortex of 1-day-old rats. These data are consistent with the putative neurotransmitter function of NPY in the nervous system[2–4] and establishes that the neurons of this co-culture system contain and release NPY. While these cultures are only 85–90% neuronal, it appears unlikely that any of the NPY was glial since pure primary glial preparations from these brains synthesize none of the peptide

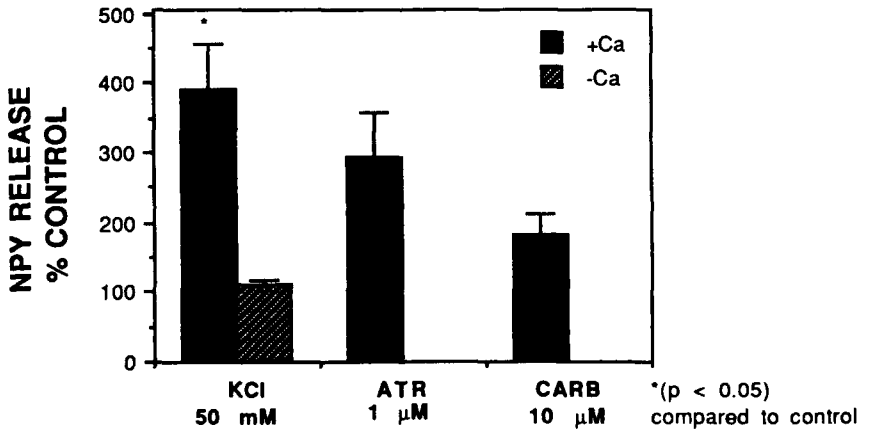

FIGURE 3. The effects of K+ depolarization and cholinergic treatments on the release of NPY from 15-day-old primary neuronal co-cultures (neocortex and basal forebrain) (Student *t* test). KCl = potassium chloride, ATR = atropine and CARB = carbachol.

(not shown). At present, however, we cannot ascribe the NPY to neocortical neurons since it is possible that some basal forebrain neurons may also have synthesized some of the peptide as well under these conditions.

Perhaps the most interesting result of this study is the ability of atropine to increase pre-pro NPY mRNA in neuronal co-cultures, suggesting that muscarinic transmission exerts a tonic inhibition on pre-pro NPY mRNA expression. It is not surprising that the muscarinic agonist carbachol had no effect under these conditions, since for atropine to be effective there must already be substantial muscarinic receptor stimulation.

The ability of forskolin to increase pre-pro NPY mRNA in primary neuronal co-cultures suggests that NPY gene transcription in these cells is stimulated by cyclic AMP accumulation. Elevation of cyclic AMP stimulates the transcription of several neuropeptide genes including NPY (28–31). NPY gene expression was shown to be positively regulated by forskolin in PC12 rat pheochromocytoma and N18TG-2 mouse neuroblastoma cell lines.[28] Therefore, our data are in agreement with others and show that NPY gene expression in primary neocortical and basal forebrain cultured neurons is also regulated by cyclic AMP.

That the tonic inhibition of mRNA synthesis by basal cholinergic transmission may be due to an inhibitory action on cyclic AMP synthesis is also suggested by the forskolin study, since if atropine were acting by some other mechanism from cyclic AMP synthesis, one would expect some additive effects when forskolin and atropine were added. This hypothesis is supported by the observation that the M_2 cholinergic receptor subtype can inhibit adenylate cyclase, while none has been found to activate adenylate cyclase. However, additional work will be necessary to characterize the muscarinic receptor subtypes in more detail.

The functional significance of the atropine-induced elevation in NPY mRNA levels in this co-culture system is unclear, since there was no corresponding elevation in NPY levels or release. In contrast, we observe that depolarization, which increases release in a calcium dependent manner, also increases mRNA synthesis and total NPY levels in a calcium dependent manner. One interesting possibility is that the regulation of NPY gene expression occurs without changes in membrane potential (suggested by the inability of atropine or carbachol to trigger NPY release), so that mRNA is synthesized for use at a later time when the NPY neuron is depolarized. This type of precursor synthesis in the cell body for subsequent use later when the nerve is activated may be a novel type of neuromodulation if the mRNA ever becomes translated. Even more interesting is the possibility that this may provide a mechanism for changes in synaptic input (muscarinic) to alter the amount of transmitter released much later from the NPY neuron. Such a long-term change in transmission in a pathway following earlier activation of the pathway may be a substrate for a working "memory."

The atropine-induced increase in NPY encoding mRNA appears to be consistent with the results of bilaterally nBM-lesioned rats. NPY levels are elevated in the parietal corticies of bilaterally nBM lesioned rats at 10, 14 and 20 months postlesioning compared to sham operated controls, but not at 2 months postlesioning.[18] However, it is very difficult to compare these *in vitro* data with the effects of cholinergic treatments or nucleus basalis lesions *in vivo*, of course, due to the possibilities that: 1) the cultures may have different synaptic connections than the brain does; 2) the cultured NPY containing neurons may express a different ratio of muscarinic receptor subtypes than the brain NPY neurons do; and 3) the NPY neurons themselves may respond differentially to the same treatments than brain neurons do because of differential receptor-transduction processes or gene regulatory processes (*e.g.*, some de-differentiation occurs). Nonetheless, it appears that at least one type of muscarinic receptor can modulate the activity of NPY encoding genes. Whether this is a direct action on the NPY neuron requires additional study, *e.g.*, measuring the effects of atropine or carbachol in the presence of tetrodotoxin, which

blocks impulse generation and the intermediary actions of neurons between cholinergic terminals and NPY neurons.

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Neuropeptide Y and Memory Processing

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Numerous neurotransmitters have been demonstrated to modulate memory (TABLE 1). Neuropeptide Y (NPY) has been demonstrated to modulate a variety of behaviors.[1] NPY is highly concentrated in the hippocampus and amygdala.[2] The highest concentrations of NPY binding sites are in the hippocampus.[3] As both the amygdala and hippocampus are known to be important areas involved in memory processing, we postulated that NPY may play a role in memory modulation.

Memory Processing and Central Injections of NPY

Posttraining intracerebroventricular (ICV) administration of NPY to mice, which were undertrained, on a step-down passive avoidance task, resulted in improved retention when the mice were re-tested 7 days later.[4] A maximum improvement in memory was seen between 0.25 and 0.5 micrograms of NPY and the dose reponse demonstrated a typical inverted-U shaped curve.

Next, the effects of NPY on memory retention administered ICV immediately after training in a T-maze active avoidance task were tested. NPY improved retention in this active avoidance task with maximum improvement occurring at a dose of 5 micrograms. NPY free acid was ineffective at improving memory in this paradigm, suggesting a specific effect of the active form of NPY. Peripheral administration of NPY had no effect on memory processing, showing that NPY was exerting its effect within the central nervous system and not by diffusing out of the brain into the periphery. The effect of NPY on memory retention was time dependent with the effect dissipating when NPY was administered 90 minutes after training indicating that the effect of NPY on retention testing was not due to a proactive effect of the peptide. NPY had no effect on acquisition of T-maze footshock avoidance confirming a specific effect of NPY on memory retention.

Previous studies have demonstrated that approximately one-tenth of the optimal dose of a memory retention enhancing agent will improve recall when administered just before retention testing.[5,6] One week after T-maze footshock avoidance training NPY was administered ICV immediately prior to retention testing. NPY enhanced retention with the optimal dose being 0.25 micrograms of NPY.[4] As NPY did not alter acquisition, this enhanced recall most probably reflects enhanced retrieval of previously stored memories.

Amnesia produced by inhibitors of protein synthesis can be reversed by numerous neurotransmitter agonists and hormones.[7] Similarly, NPY was shown to reverse amnesia produced by the protein synthesis inhibitor, anisomycin (20 mg/kg).[4] Scopolamine is an acetylocholine receptor antagonist. Scopolamine induces amnesia. Scopolamine-induced

amnesia is reversed by a variety of neurotransmitters.[7] Mice receiving saline had an 80% recall score; scopolamine treated mice had only a 13% recall score demonstrating the amnestic effect of scopolamine. Mice receiving both scopolamine and NPY had an 80% recall score, demonstrating the ability of NPY to reverse the scopolamine-induced amnesia.[4]

Recently food ingestion has been demonstrated to enhance memory retention for a T-maze aversive test.[8] NPY is a potent anorexigenic agent.[9] This suggested that the memory enhancement produced by NPY could be secondary to its ability to increase food intake. To test this possibility, we tested the ability of NPY to enhance memory when food was not available for 3 hours after training. NPY was equally effective at enhancing memory retention when food was not available.[4] Therefore, the NPY effect on memory retention is a primary one and not secondary to its ability to enhance food intake.

Administration of optimal memory retention enhancing doses of cholinergic agonists such as arecoline and tacrine impair retention when mice are well (over) trained rather than under (poorly) trained. Other neurotransmitters that enhance memory retention will impair memory retention in well trained mice. NPY is no exception to this rule. When mice were given 5 trials instead of 4, footshock was increased from 0.30 to 0.35 mA and the buzzer was louder (65dB compared to 55dB), 73% of controls remembered the task

TABLE 1. Neurotransmitters Known to Modulate Memory

Memory Enhancers	Amnestic Agents
Acetylcholine	Gama-amino butyne acid
Alpha-noradrenergic	Beta-endorphin
Serotonin	Vasoactive intestinal peptide
Dopamine	
Somatostatin	
Cholecystokinin	
Vasopressin	
ACTH	
Neuropeptide Y	
Neuropeptide K	

compared to 20% or less in the undertrained condition. NPY administered ICV at a dose of 5 micrograms impaired retention, with a recall score of only 13%.[4] Thus NPY follows the general rule for memory retention enhancing drugs, *i.e.*, that these agents are amnestic in overtrained animals.

To summarize, NPY when administered ICV, is a potent memory enhancing agent. NPY demonstrates many parallels to the memory enhancement seen with the cholinergic agent, arecholine (TABLE 2).

Site of Action

To examine the forebrain sites at which NPY produced its effect on memory retention we injected 0 to 1 microgram NPY or saline in a volume of 0.5 microliters into the caudal and rostral portion of hippocampus, the septum, the amygdala, the caudate and the thalamus.[10] NPY, at an optimum dose of 0.5 μg, enhanced memory for the T-maze footshock active avoidance task when injected into the rostral hippocampus and the septum. NPY was amnestic when injected into the amygdala and caudal hippocampus. Injections of NPY into the caudate and thalamus were without effect. Injection of NPY into cortical sites just above the rostral hippocampus and septum also failed to produce an

TABLE 2. Comparison between the Effects of Neuropeptide Y and Arecoline (NPY) on Memory

	NPY	Arecoline
Acquisition	no effect	no effect
Memory retention	enhanced	enhanced
Reverses anisomycin amnesia	yes	yes
Reverses scopolamine amnesia	yes	yes
Memory recall	enhanced	enhanced
Enhanced memory in old mice	yes	yes
Amnesia in overtrained mice	yes	yes

effect on memory retention. This confirmed the specificity of the sites of action at which NPY modulates memory retention.

The physiologic role of NPY on memory retention was tested by passively administering NPY antibodies into these structures that had been shown to be NPY sensitive. NPY antibodies were amnestic when injected into the rostral hippocampus and septum and enhanced memory retention when injected into the caudal hippocampus and the amygdala.[10] These effects of NPY antibody administration were opposite to those obtained with NPY administration.

To further examine the specificity of the effect of NPY, we examined the effect of localized injections of a similar size neuropeptide, Neuropeptide K. NPK is a 36-amino acid peptide which contains the sequence of the substance P presursor, neurokinin A, as amino acids 27 to 36 of its C-terminus. Neuropeptide K is present in high concentrations in the hippocampus,[11] and neurokinin A receptors are present in the hippocampus and the amygdala.[12] Both NPK and neurokinin A have been shown to enhance memory for the aversive T-maze paradigm when administered intracerebroventricularly immediately after training.[13] TABLE 3 compares the effects of localized injections of neuropeptide K to those of NPY. Like NPY, neuropeptide K enhanced memory when injected into the rostral hippocampus. However, neuropeptide K also enhanced memory in two areas where NPY was amnestic, *viz*, the caudal hippocampus and amygdala. Neuropeptide K produced no effects in the septum, an area in which NPY enhanced memory retention. These studies show that NPY and NPK have specific sites within the central nervous system at which they modulate memory and that the memory modulating effects of 36-amino-acid peptides are not due to nonspecific effects of peptides of this size.

Overall this series of studies has demonstrated that NPY is a physiological modulator of memory. In addition, the effects of NPY on memory are dependent on the anatomic site at which it is released. This anatomic specificity supports the concept that NPY is acting

TABLE 3. Comparison of the Effects of Localized Injections of Neuropeptide Y (NPY) and Neuropeptide K (NPK) on Memory Retention

	Effect on Memory	
Anatomic Site	NPY	NPK
Hyppocampus-rostral-caudal	enhanced	enhanced
	amnestic	enhanced
Amygdala	amnestic	enhanced
Septum	enhanced	no effect
Thalamus	no effect	no effect

as a neuromodulator, perhaps through modulating the release or receptor function of a co-released classical neurotransmitter.

NPY Receptors Memory and Feeding

NPY is the most potent orexigenic agent yet to be discovered.[14] It produces its effects on feeding predominantly in the ventromedial hypothalamus and in the paraventricular nucleus of the hypothalamus,[9] as well as in structures associated with the fourth ventricle.[14]

Recently it has been demonstrated that there are two classes of NPY receptors; a postsynaptic (Y_1) receptor and a presynaptic (Y_2) receptor. Shorter NPY fragments bind only to the Y_2 receptor. Y_2 receptors are present in neuronal membranes of the hippocampus.[15] Our studies showed that the shorter NPY segments were capable of producing memory enhancement.[16] On the other hand, only the intact fragment was capable of producing food ingestion. Thus the memory effects of NPY appear to involve

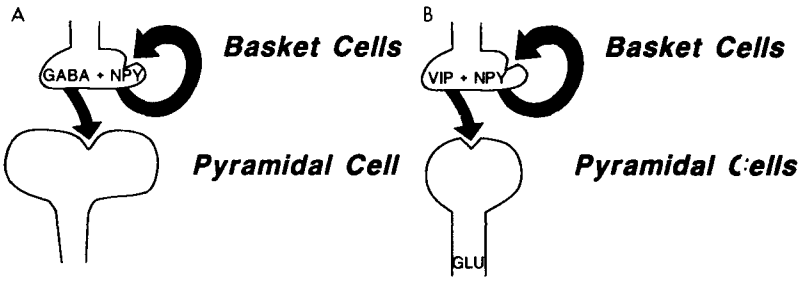

FIGURE 1. **(A)** Neuropeptide Y (NPY) could be co-released with gamma amino butyric acid (GABA) and NPY could feedback presynaptically on the basket cell to inhibit further release of GABA. Thus GABA would no longer inhibit the firing of the pyramidal cell containing glutamate. Glutamate enhances memory retention. **(B)** A similar scenario is postulated for NPY inhibiting the release of vasoactive intestinal peptide (VIP). Alternatively VIP could act on a separate set of pyramidal cells.

the presynaptic (Y_2) receptors, while the feeding effects utilize postsynaptic (Y_1) receptors.

Mechanism by Which NPY Enhances Memory

The activity of classical neurotransmitter activity resulting from an action potential can be modified by coexisting classical neurotransmitters. NPY is found in basket-type cells in the hippocampus that synapse on perikarya and dendrites of pyramidal cells in the hippocampus.[17] In general, the major neurotransmitter in basket cells is gamma amino butyric acid (GABA).[18] Application of NPY to the hippocampus reduces spike amplitude when this is recorded either extracellularly or from pyramidal cells;[19,20] thus suggesting its effects are mediated by an interneuron. NPY fails to alter the excitatory effect of glutamate applied iontophoretically to the hippocampus.[19] As NPY acts predominantly on the presynaptic (Y_2) receptor to enhance memory, we suggest that NPY produces its memory enhancing effect by inhibiting release of GABA from basket cells, and thus facilitating firing of glutamate containing pyramidal cells (FIG. 1A).

Vasoactive intestinal peptide (VIP) is a potent amnestic agent when injected directly into the rostral portion of the hippocampus (unpublished observations). This effect of VIP is not reversed by co-administration of NPY, but is reversed by the cholinergic analog, arecholine. FIGURE 1B gives a scheme by which NPY would be co-released with GABA, and VIP would be released from a separate basket cell. Alternatively, all three neurotransmitters could be co released.

These studies allow the beginnings of an understanding of how classical neurotransmitters and neuropeptides might interact to modulate memory.

Alzheimer's Disease and Neuropeptide Y

Hippocampal neurons and axons containing immunoreactive NPY are involved in plaque formation in Alzheimer's Disease.[21,22] NPY is present in the same cell bodies as somatostatin and these cell bodies ar involved in the pathological processes of Alzheimer's disease.[23] Measurements of immunoreactive NPY have suggested a reduced level in cerebral cortical cells of patients with Alzheimer's disease,[24] but other studies have failed to show changes in cortical cells.[25,26] Increased levels of immunoreactive NPY have been found in the substantia innominata of patients with Alzheimer's disease.[27] Overall it appears that changes in immunoreactive NPY occur in Alzheimer's disease or only in some forms of Alzheimer's disease. Cerebrospinal fluid levels of immunoreactive NPY were reduced by 18% in patients with dementia associated with extrapyramidal signs but not in those with dementia not associated with extrapyramidal signs.[28] These studies suggest a possible role for NPY in the pathogenesis of Alzheimer's disease.

CONCLUSION

Memory retention appears to be under the regulation of numerous neurotransmitters. Many of these seem to play a modulatory role in allowing the development of the memory trace. NPY appears to be a potent physiological modulator of memory. The effects of NPY are anatomically specific. Within the rostral hippocampus NPY may exert its effects by being co-released with GABA from basket cells in the hippocampus. NPY would then feedback on presynaptic receptors (Y_2) to inhibit the release of GABA. Lack of release of GABA would allow the unimpeded release of the memory enhancing neurotransmitter, glutamate. Conflicting results exist concerning the effect of Alzheimer's disease on NPY levels measured by radioimmunoassay. It is possible that an imbalance of NPY together with other neurotransmitters may play a role in the pathogenesis of Alzheimer's disease.

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PART IV. FUNCTIONS OF NEUROPEPTIDE Y IN THE HYPOTHALAMUS

Ultrastructural Localization of Neuropeptide Y in the Hypothalamus

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid C-terminally amidated peptide of the pancreatic polypeptide family.[1,2] Both the gene[3,4] and mRNA[5-7] sequences for rat and human NPY have been determined and predict a precursor consisting of a signal peptide followed by NPY and a C-terminal peptide. NPY is found widely distributed and in high concentrations in the mammalian brain, particularly within hypothalamus, hippocampus and cortex.[8,9] The distribution of NPY immunoreactivity in the hypothalamus of different species has been extensively studied by means of immunocytochemistry mostly performed at the light microscopic level.[8-13] In all the species studied, the highest concentration of NPY-containing cell bodies has been found in the arcuate nucleus. In the rat, immunoreactive cell bodies have also been found in smaller amounts in the paraventricular nucleus, suprachiasmatic nucleus and preoptic area. NPY fibers are most densely packed in the paraventricular nucleus, suprachiasmatic nucleus and preoptic area. Lower densities of NPY-containing fibers are observed in the anterior hypothalamic area, periventricular nucleus, arcuate nucleus, ventromedial and dorsomedial nuclei, and the inner zone of the median eminence.

Evidence is accumulating to indicate that NPY is implicated in neuroendocrine functions[14] as well as other physiological activities, including food intake[15] and cardiovascular function.[16] In order to better understand the role as well as the regulation of the hypothalamic NPY system, it is required to determine the connections of this system with the other monoaminergic and peptidergic hypothalamic systems. These studies require precise localization of the different systems at the ultrastructural level. In the present paper, we will present data obtained by our group on the ultrastructural localization of NPY as well the anatomical connections between NPY and monoaminergic neurons.

METHODS

Immunocytochemical Localization of NPY

In all the experiments, adult male rats weighing 250–350 g were used. For light microscopic studies, a few animals received an injection of 30 μg of colchicine into the left lateral ventricle 48 hours before fixation.

For preembedding staining, they were perfused with 200 ml of a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Consecutive coronal sections (30–40 μm) were cut with the Vibratome (Oxford). The staining was performed on the Vibratome sections using the peroxidase-antiperoxidase (PAP)

complex as described by Pickell *et al.*[17] Some sections were mounted on glass slides for light microscopic observations whereas others were postfixed in osmium tetroxide, dehydrated in ethanol and flat-embedded in Araldite.

For postembedding staining, the animals were fixed by perfusion with 200 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Several hypothalamic areas were carefully dissected, postfixed in 2% osmium tetroxide, dehydrated in ethanol and embedded in Araldite. Ultrathin sections were mounted on nickel grids and were immunostained with the immunogold staining method involving use of colloid gold coated with protein A.[18]

In these studies, the antiserum (no 820) to NPY was used at a dilution ranging from 1:500 (for preembedding staining) to 1:800 (for postembedding staining). This antiserum showed no significant cross-reactivity with either PYY or APP.[12] Specificity of the immunostaining was further determined by absorption of the antiserum (diluted at 1:500 and 1:800) with the following antigens at a final concentration of 10^{-6} M: NPY, PYY, avian PP (APP), somatostatin, LHRH and β-endorphin. Only immunoabsorption with NPY could prevent staining, all the other peptides being totally ineffective in blocking reaction.

Simultaneous Localization of NPY and Tyrosine Hydroxylase

The animals were fixed by perfusion with 200 ml of 4% paraformaldehyde at pH 6.5 in sodium acetate buffer and then with 4% paraformaldehyde at pH 10.5 in carbonate-bicarbonate buffer as described by Berod *et al.*[19] The brains were dissected and postfixed at 4°C in the same alcaline fixative. Using Vibratome, serial sections (50 μm) were obtained. The immunocytochemical staining was performed with the anti-NPY antiserum and an anti-tyrosine hydroxylase (TH) antiserum (Institut Jacques Boy S.A., France). The double-labelling procedure consisted firstly in the incubation of sections with the primary antiserum diluted 1/1000 in 0.05 M Tris Buffer (TB), pH 7.6, overnight at room temperature followed by an 1-hour incubation with goat anti-rabbit-γ-globulins diluted 1/50 in TB and then incubation in the peroxidase-antiperoxidase (PAP) complex diluted 1/50 in TB for 30 min. The reaction was revealed with diaminobenzidine (DAB) and H_2O_2 for 5 min. After washing in water and TB, sections were then incubated in the second primary antiserum overnight at room temperature followed by 1.5 hour incubation with goat anti-rabbit-γ-globulins. The intensification of the first DAB reaction was done at this step. Sections were washed in a solution of 2% sodium acetate for 15 min. They were then incubated in a solution of 10% thyoglycolic acid for 2 hours and washed again in 2% sodium acetate and developed during 10–20 min. The developer was prepared as described by Gallyas *et al.*[20] After intensification, the second primary antiserum was revealed by incubation overnight in PAP complex at room temperature. After the DAB reaction, the sections were postfixed in 2.5% glutaraldehyde for 15 min, washed in phosphate buffer 0.1 M, pH 7.4, containing 5% sucrose, fixed again in 1% osmium tetroxide for 1 hour, and then dehydrated and embedded in Araldite. Semithin and ultrathin sections were respectively mounted on glass slides or grids. The ultrathin sections were contrasted with uranyl acetate and lead citrate. As control, we used in alternate the anti-TH or anti-NPY antiserum in the first sequence of staining. Whatever the sequence of staining, the results were identical.

RESULTS AND DISCUSSION

Localization of NPY in the Rat Hypothalamus

Observation of the Vibratome sections revealed that in colchicine-treated animals a high concentration of immunostained cell bodies could be detected in the arcuate nucleus

FIGURE 1. Coronal section through the mediobasal hypothalamus of a colchicine-treated rat. Neuronal cell bodies (→) staining for NPY are abundant in the arcuate nucleus. Positive fibers can also be observed. V: third ventricle. X180.

(FIG. 1). Less numerous stained neurons were also observed in the periventricular and paraventricular nuclei. In nontreated rats, the stained fibers were more numerous than in colchicine-treated animals. They were found in high concentrations in the arcuate, paraventricular, periventricular and suprachiasmatic nuclei (FIG. 2) and in moderate

amounts in the dorsomedial and ventromedial nuclei, and in the inner zone of the median eminence (FIG. 3).

At the electron microscopic level, with the preembedding technique involving use of PAP, immunostaining was observed in nerve fibers in the periventricular nucleus, paraventricular nucleus, suprachiasmatic nucleus and arcuate nucleus. In all these areas, the staining pattern was very similar. NPY-immunoreactivity was observed in both dendrites and axons. In the dendrites, the staining was rather diffuse and not associated with any specific organelles. In the axon profiles and endings, the staining was mostly detected in the large dense core vesicles (70–100 nm in diameter) and also more diffusely throughout the axoplasm (FIG. 4). The small clear vesicles seemed devoid of reaction product which formed a ring of labelling around their external membranes. So far, immunostaining has always been observed in unmyelinated but not in myelinated processes.

In areas reported to contain NPY-immunoreactive cell bodies, such as the paraventricular, periventricular and arcuate nuclei, immunostained cell bodies could be detected. The staining was localized primarily in dense core vesicles (70–100 nm in diameter) but also distributed more diffusely throughout the cytoplasm (FIG. 5).

With the immunogold technique, NPY immunostained neuronal cell bodies and axons were also observed in the periventricular, paraventricular and arcuate nuclei of the hypothalamus. No stained dendrites could be detected with this technique. However, in the immunoreactive cell bodies and axons, the immunogold labeling was restricted to the

FIGURE 2. Section through the anterior hypothalamus. Numerous fibers staining for NPY are present in the ventral portion of the suprachiasmatic nucleus (→). V: third ventricle. X200.

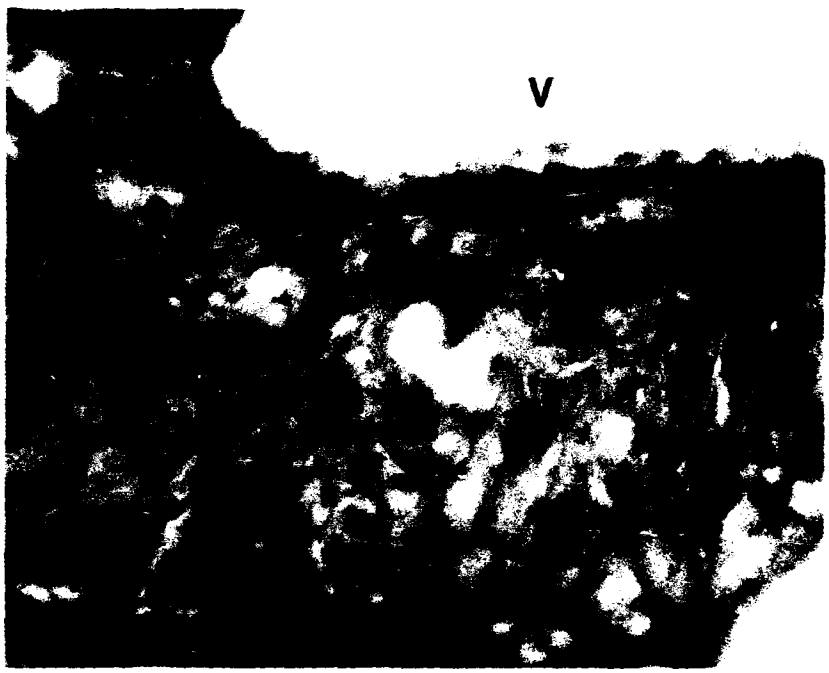

FIGURE 3. Rat median eminence. Immunostained fibers are observed in the inner zone (IZ). V: third ventricle. X450.

large dense core vesicles (70–100 nm in diameter) (FIGS. 6 and 7). As observed with the preembedding technique, the small clear vesicles (30–50 nm in diameter) were never found to be stained.

Both techniques provided excellent ultrastructural preservation, thus allowing the identification of synaptic contacts and the evaluation of NPY-immunoreactive terminals forming typical synaptic junctions. On 74 labelled terminals found in the periventricular nucleus, arcuate nucleus and suprachiasmatic nucleus, about 20% of them were observed forming asymmetrical or symmetrical synaptic contacts with nerve processes, especially dendrites. Most of the other terminals appeared as free endings without typical synaptic contact.

Anatomical Connections between NPY and Serotonin (5-HT) Systems

Suprachiasmatic Nucleus

In the suprachiasmatic nucleus, a large number of both 5-HT and NPY afferent fibers overlap in the ventral part of the nucleus.[21] This localization suggests interactions between the two systems which might be involved in the regulation of circadian rhythms. In order to investigate the possible anatomical interactions between these two systems, experiments combining [^{3}H]5-HT autoradiography and NPY immunocytochemistry were conducted at the ultrastructural level.[22]

It was demonstrated that NPY and 5-HT were not co-localized in the same structures.

Both NPY and 5-HT terminals were shown to be in synaptic contacts, sometimes on the same neuronal target. Some cases of direct axoaxonic appositions between the two types of terminals were also observed. These appositions did not involve the typical membrane specialization which characterizes the true synaptic contacts. These morphological observations suggest a possible physiological interaction between these two systems which remains to be elucidated.

Arcuate Nucleus

The arcuate nucleus which contains the highest concentration of NPY neurons received an important 5-HT innervation[21] suggesting the possibility of interactions between the two systems. In fact, connections between 5-HT fibers and dopaminergic and proopiomelanocortin (POMC) neurons have already been demonstrated in the arcuate nucleus.[23] Using a combination of autoradiography (5-HT localization) and immunocytochemistry (NPY localization), Guy *et al.* [24] have studied the 5-HT/NPY interactions. Direct nonsynaptic appositions between [^{3}H]5-HT labeled terminals and immunoreactive cell bodies or dendrites, also receiving unlabelled afferents, could be observed. Occasionally, axoaxonic appositions between [^{3}H]5-HT labelled and NPY immunoreactive profiles were detected. Considering that NPY neurons are projecting to the paraventricular and dorsomedial nuclei of the hypothalamus,[11,25] it can be hypothesized that arcuate 5-HT/NPY interactions could be involved in the 5-HT and/or NPY-mediated regulation of feeding behavior[15] or neuroendocrine functions.[14]

Anatomical Connections between NPY and Catecholaminergic Systems

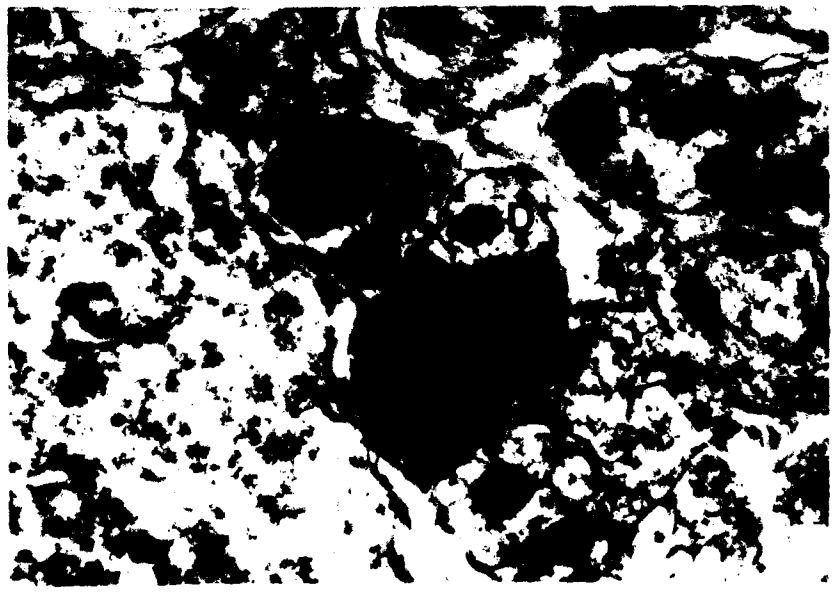

FIGURE 4. Preembedding NPY staining in the arcuate nucleus. A positive ending (→) is in synaptic contact with an unlabelled dendrite (D). X35,000.

FIGURE 5. Preembedding staining for NPY in the arcuate nucleus. A neuronal cell body is immunostained. Note the presence of strongly immunoreactive dense core vesicles (→). RER: rough endoplasmic reticulum: N: nucleus. X26,000.

shown[27] a marked increase in NPY immunoreactivity in the arcuate nucleus in rats which had been treated with α-methylparatyrosine (an inhibitor of catecholamine synthesis) or haloperidol (a dopaminergic antagonist) (FIG. 8). Vallejo *et al.*[28] have demonstrated that

intracerebroventricular administration of NPY decreased noradrenaline turnover in the hypothalamus as well as in the brainstem and in the striatum. Härfstrand *et al.*[26] have shown, by immunocytochemistry, a substantial overlap between the distribution of tyrosine hydroxylase (TH) immunoreactive nerve terminals and the NPY immunoreactive nerve terminals within the ventrolateral part of the arcuate nucleus. In order to provide ultrastructural evidence for such NPY/CA interactions in the arcuate nucleus, we have used a double immunostaining procedure at both light and electron microscope levels (see Methods).[29]

FIGURE 6. Immunogold labelling in the arcuate nucleus. In a positive cell body, the dense core vesicles are decorated with gold particles (→). N: nucleus, M: mitochondria. X38,000.

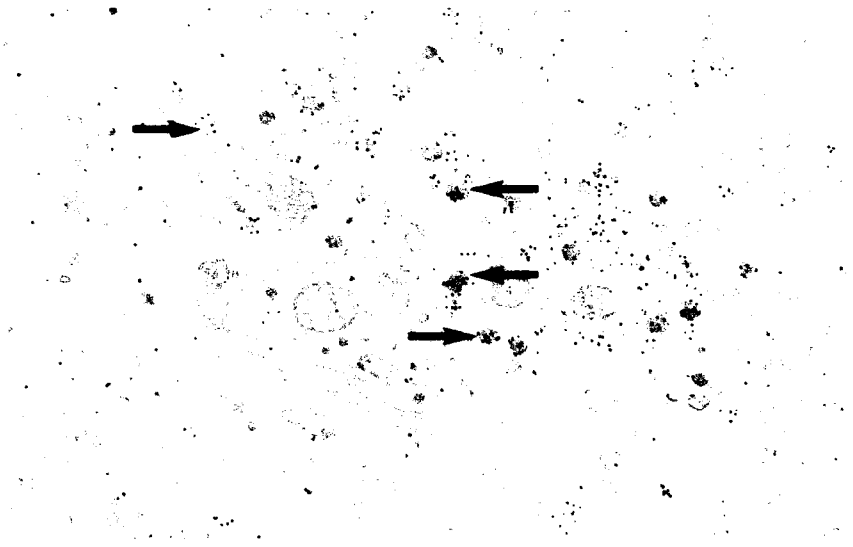

FIGURE 7. *Immunogold labelling in a median eminence section. Two positive free endings can be observed in the inner zone. The gold particles are overlying the dense core vesicles (→). ×32,000.*

In the double-stained preparation observed at the light microscopic level, the first antiserum was intensified by silver-gold which produces a black staining and the second was stained by the DAB chromogen only giving the classical brown coloration. In semithin sections, we observe TH-immunoreactive terminals in proximity to NPY immunostained cell bodies and also NPY-immunoreactive terminals very close to TH-immunoreactive cell bodies (FIG. 9). In addition, NPY cell bodies were seen in close proximity to other NPY-immunopositive structures.

At the electron microscopic level, two types of immunostained structures were easily detectable. The intensifed reaction was characterized by aggregates of gold particles and the second immunohistochemical staining appears as a diffuse electron density as observed in the preembedding staining. All types of elements including cell bodies, axons, and dendrites were immunostained either for NPY or TH. In this study, we have seen just a few direct appositions between TH- and NPY-immunoreactive structures. These appositions were of the axodendritic or axosomatic types such as a NPY-immunopositive terminal on a TH-immunoreactive dendritic cell (FIG. 10) or a TH immunoreactive terminal on a NPY-immunoreactive cell body (FIG. 11). A few axoaxonic appositions were also seen. As it was suggested by light microscopic observations, we saw some direct appositions between two NPY-immunoreactive structures (FIG. 11). At all these axosomatic, axodendritic and axoaxonic contacts, no synaptic membrane differentiation could be observed, whereas morphologically defined synapses could be routinely observed in adjacent areas in the same preparation. Since we have used a TH antiserum to label cathecolamines, it is then not possible to determine whether TH immunopositive fibers contain dopamine, noradrenaline or adrenaline since the arcuate nucleus is known to contain the three types of catecholaminergic innervations.[30] By contrast, TH immunopositive cell bodies and dendrites, within the arcuate nucleus are exclusively dopaminergic[30] and belong to the A-12 dopaminergic tuberoinfundibular neurons.[31]

Our results showed direct appositions between TH and NPY immunoreactive struc-

tures and also between two NPY immunoreactive structures. The fact that we had no synaptic membrane differentiation could not be attributed to an inadequate fixation since such densities could be observed in adjacent areas. The low sensitivity of this combined

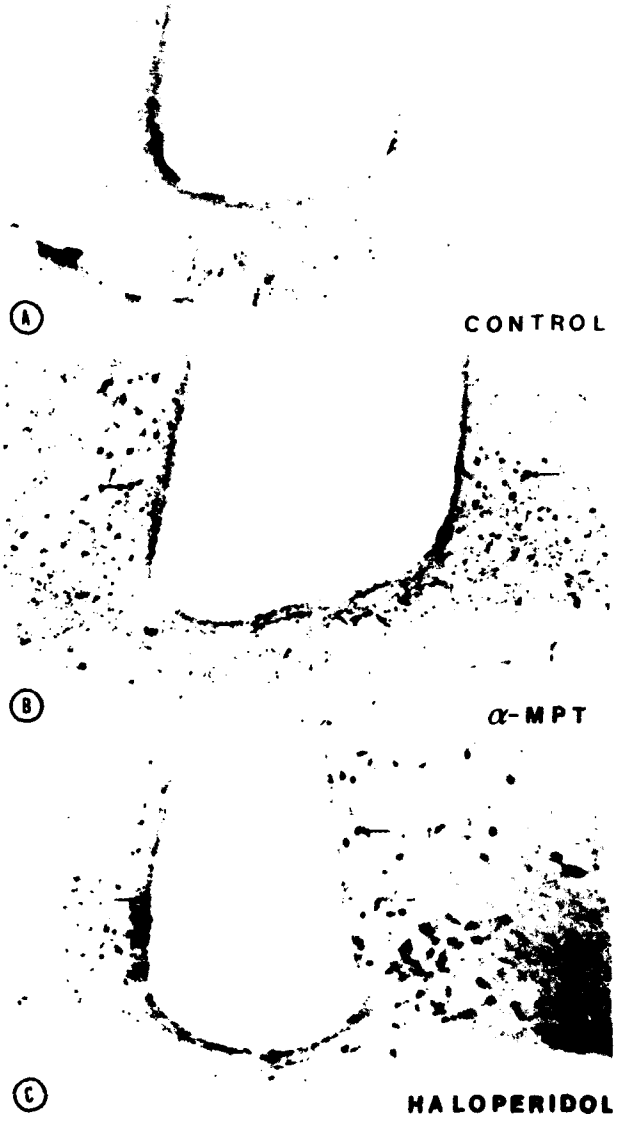

FIGURE 8. Sections through the arcuate nucleus. **(A)** Control rat; **(B)** rat treated with α-MPT; **(C)** rat treated with haloperiodol. NPY neuronal cell bodies (→) are absent in control rat and numerous in haloperidol and α-MPT-treated animals. X140.

FIGURE 9. Micrograph of a semithin plastic section obtained after double staining. TH-immunopositive fibers which have been silver-gold intensified and appear black (→) are in very close proximity to a NPY-immunoreactive cell body. Note the presence of some other immunostained structures intensified or not. X2000.

staining could, however, decrease the possibility of seeing some of these membrane specializations between TH and NPY neurons. This could also explain why we have not observed synaptic contacts between two TH immunoreactive structures. Moreover, these TH synaptic contacts were not shown by Piotte *et al.*[32] who described the presence of unlabeled terminals synapsing on TH labeling dendrite. However, in the present study, the lack of synaptic contact could be explained by the presence of gold particles on immunoreactive structures which might have a masking effect on synaptic membranes. A reconstruction study could help to determine the presence or the absence of synaptic junctions. In the literature, there are other examples of nonsynaptic cellular interaction in the arcuate nucleus. Such cases include proopiomelanocortin POMC/POMC,[33] 5-HT/dopamine,[23] 5-HT/POMC,[23] DA/endorphin[34] and recently 5-HT/NPY[22,24] interactions.

In conclusion, these morphological data showing direct appositions between TH immunoreactive structures and NPY containing neurons in addition to the pharmacological

data obtained by our[27] and other groups,[26,28,35] strongly suggest that there is a direct interaction between these two systems in the arcuate nucleus.

SUMMARY AND CONCLUSIONS

Immunoreactive NPY neurons are widely distributed in the hypothalamus of several mammalian species. In the rat, dense NPY fiber networks are found in the paraventricular, suprachiasmatic and arcuate nuclei. NPY-containing cell bodies are mostly found in the arcuate nucleus. Studies performed at the electron microscope level clearly indicate that NPY is concentrated in dense core vesicles in the cytoplasm of cell bodies as well as in terminals. Only a small percentage (about 20%) of the NPY endings are making

FIGURE 10. Electron micrograph showing a NPY-immunoreactive terminal (→) which had been silver-gold intensified in direct apposition to a TH-immunopositive dendritic element. Note the asymmetrical synapse of an unlabelled structure (*). X45,0000.

FIGURE 11. Electron micrograph showing a TH-immunoreactive profile (gold particles) which is in direct apposition to an NPY-immunoreactive (NPY) cell body. A NPY-immunopositive terminal (*) is directly apposed to the same NPY cell body. The nucleus (Nu) is completely negative. X33,0000.

synaptic contacts with nerve processes, especially dendrites. These ultrastructural data suggest that NPY might play a neurotransmitter/neuromodulator role.

NPY has been shown, when injected into hypothalamic areas, to exert a variety of effects, including modifications in food intake, energy balance and pituitary secretion. In an attempt to define the exact role of NPY in hypothalamic functions, we have designed

experiments to study the interactions of NPY with other neurotransmitter systems. In the suprachiasmatic nucleus, both NPY and 5-HT terminals have been shown to establish synaptic junctions sometimes with the same neurons. Occasionally, axoaxonic junctions between these two types of endings have been observed. These results suggested that both 5-HT and NPY might be involved in the complex regulation of circadian rythms. In the arcuate nucleus, nonsynaptic appositions between 5-HT nerve endings and NPY-containing neurons were demonstrated. In this nucleus, direct appositions between TH- and NPY-containing neurons were also detected. These appositions were of axosomatic, axodendritic or axoaxonic types. Since it has been demonstrated that arcuate NPY neurons are projected to other hypothalamic areas, such as the paraventricular and dorsomedial nuclei, it might be speculated that arcuate 5-HT/NPY and catecholamines/NPY interactions might be involved in regulation of behavior and neuroendocrine functions.

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Neuropeptide Y in the Circadian Timing System[a]

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The Circadian Timing System

Circadian rhythms are adaptations to the solar cycle of light and dark. In mammals, circadian rhythms express a temporal organization of physiological processes and behavior that results in optimal animal adaptation. The essential features of circadian rhythms are that they are generated by endogenous pacemakers and continuously reset, a process termed "entrainment," by the light-dark cycle. This generation and entrainment of circadian rhythms is accomplished by a set of central neural structures, the circadian timing system (CTS). The principal components of the CTS are visual pathways mediating entrainment, pacemakers and efferent projections from the pacemakers to effector systems that exhibit circadian function (FIG. 1).

Over the last twenty years there has been a rapid accumulation of information about the organization and function of the CTS (cf. Rusak and Zucker[1] and Meijer and Rietveld[2] for reviews that reflect this progress). The essential features of the CTS, as we now understand it, are as follows. The lateral eyes are necessary for entrainment. Light activates retinal photoreceptors which are coupled through the usual retinal circuitry to a specific set of ganglion cells, probably a subgroup of W cells,[3] responding predominantly to changes in luminous flux. These cells appear to project in both the lateral geniculate complex and to the suprachiasmatic nuclei (SCN) of the hypothalamus.[4,5] The projection to the SCN has been designated the retinohypothalamic tract (RHT). Early studies using the autoradiographic tracing method[6-8] found only projections to the SCN but more recent studies, using sensitive anterograde lectin transport methods, have shown projections to the anterior hypothalamus, lateral hypothalamus and retrochiasmatic area as well as to the SCN.[9] The function of the projections beyond the SCN is unclear at this time but there is substantial evidence to establish that the RHT to the SCN is sufficient to maintain entrainment in the absence of other visual projections.[10,11]

The SCN is a circadian pacemaker (FIG. 2). Three lines of evidence support that conclusion. First, ablation of the SCN results in a loss of circadian rhythms.[1,12,2] There are individual studies from which it is concluded that one or another rhythm, usually the

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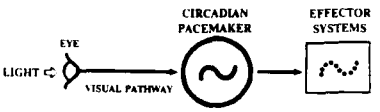

FIGURE 1. Diagram showing the necessary components of the circadian timing system. See text for description.

temperature rhythm, is maintained after SCN ablation but other studies report the same rhythm lost. It seems likely that these failures to demonstrate loss of a rhythm reflect incomplete lesions rather than the presence of non-SCN circadian pacemakers. Nevertheless, these studies have been used to support the view that the CTS is a multioscillator system containing circadian pacemakers other than the SCN.[12,13] Two other sets of data have been similarly interpreted. One is the phenomenon of internal desynchronization. This is exemplified by the "splitting" of the circadian rhythm in locomotor activity that is observed in hamsters after prolonged exposure to continuous light.[14,15] Recent evidence indicates that this reflects a desynchronization of groups of neurons within the SCN rather than the dissociation of the SCN from another oscillator.[16] Two other situations provide more compelling evidence for a non-SCN circadian pacemaker. These are the induction of a circadian activity rhythm in arrhythmic animals with SCN ablation, either by chronic administration of methamphetamine[17] or by imposition of a very restricted access to food.[18] In the latter case, in particular, the evidence that function of a non-SCN circadian pacemaker induced by the environmental stimulus is quite strong. However, in each case the pacemaker function does not persist after removal of the inducing stimulus indicating that it is a weak pacemaker and raising the question that it might not function under normal conditions. Thus, all of the ablation data are consistent with the view that the SCN is the principal circadian pacemaker.

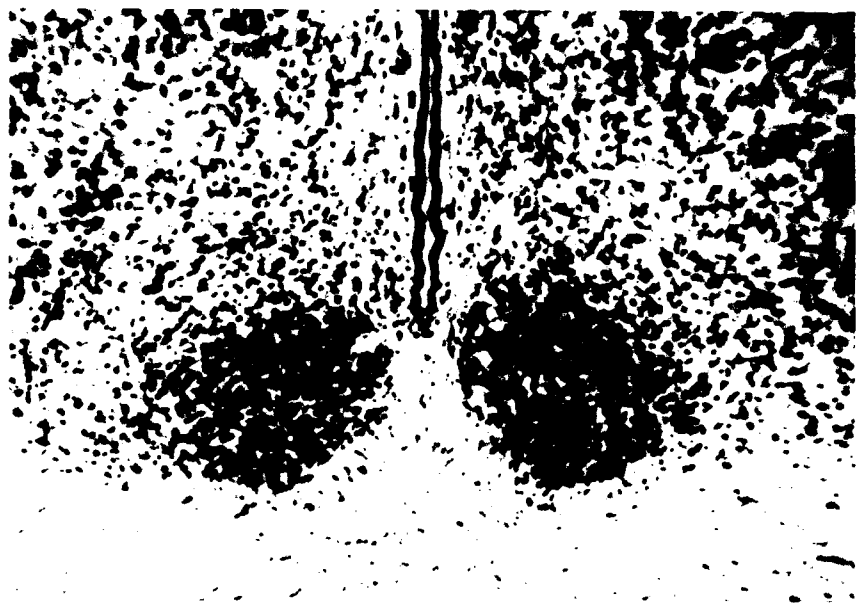

FIGURE 2. Photomicrograph of a coronal section through the rat hypothalamus showing the suprachiasmatic nuclei as two dense cell accumulations above the optic chiasm and adjacent to the third ventricle.

The second major line of evidence that the SCN is a circadian pacemaker comes from electrophysiological studies and studies of glucose utilization. In a series of elegant studies, Inouye and Kawamura[19,20] demonstrated persistence of a rhythm in multiunit activity recorded from the SCN, isolated from the remainder of the brain by knife cuts, in animals that were rendered arrhythmic by the lesion. This work was extended shortly thereafter by several investigators who demonstrated a rhythm in single unit activity in SCN neurons *in vitro* in hypothalamic slices.[21-23] Like the rhythm in multiunit activity, that in single unit activity is high in the daytime and low at night, a pattern that conforms to the pattern of SCN glucose utilization both *in vivo*[24] and *in vitro*.[25,26]

The third line of evidence is derived from studies of the effects of transplants on circadian function in animals rendered arrhythmic by SCN ablation. In these studies, the circadian locomotor activity rhythm is restored by transplants of fetal anterior hypothalamus containing the SCN[27-29] but not by transplants of other areas of fetal brain.[29] Subsequent studies using a mutant hamster with alterations of circadian period[30] have demonstrated that the restoration of rhythmicity is a function of the transplanted tissue, not an induction of remaining circadian function from the host.[31] That is, the period of the restored rhythmicity reflects the fetal donor rather than the host period. These lines of evidence clearly establish that the SCN is a circadian pacemaker in the mammalian brain.

The projections of the SCN were originally described using the autoradiographic tracing method,[32-34] and those data have been confirmed and extended using lectin transport.[35] Surprisingly, given the wide range of functions under circadian control, the projections of the SCN are very limited. The major projections are hypothalamic and largely confined to anterior hypothalamic structures in the immediately vicinity of the SCN. There also are limited projections to the tuberal hypothalamus, basal forebrain, midline thalamus and perlaqueductal gray. The very restricted distribution of SCN projections raises the issue of how pacemaker function is coupled to effector systems that exhibit circadian function, but that is beyond the scope of this review.

NPY in the Circadian Timing System

Organization of the Intergeniculate Leaflet

The lateral geniculate complex is the principal thalamic nucleus receiving retinal afferents.[36] Until recently, the complex was believed to have two major components, a dorsal lateral geniculate nucleus (DLG) derived from dorsal thalamus and projecting to the visual cortex and a ventral lateral geniculate nucleus (VLG) derived from ventral thalamus and projecting to a series of subcortical structures including the SCN.[37,38] This organizational framework was altered by Hickey and Spear,[39] who demonstrated bilateral retinal projections to a zone lying between the DLG and VLG in a pattern quite distinct from the retinal projections to either DLG or VLG. On this basis, and because of the location and shape of this new retinorecipient zone, Hickey and Spear[39] designated this region the intergeniculate leaflet (IGL). The functional significance of the IGL was not appreciated until Card and Moore[40] demonstrated that it contained NPY neurons projecting to the SCN. The presence of NPY neurons in the IGL was confirmed by Mantyh and Kemp,[41] who also described a separate population of enkephalin (ENK)-containing neurons.

The most rostral component of the geniculate complex to appear on the lateral, dorsal border of the thalamus is the DLG. As the DLG becomes prominent, NPY neurons of the IGL appear on its ventral surface and, more caudally, the VLG forms beneath the IGL. At middle levels of the geniculate complex, the IGL is a thin lamina of cells intercalated between the DLG and VLG (FIG. 3). More caudally, as the medial geniculate forms, the IGL extends medially and ventrally between the VLG and the medial geniculate to

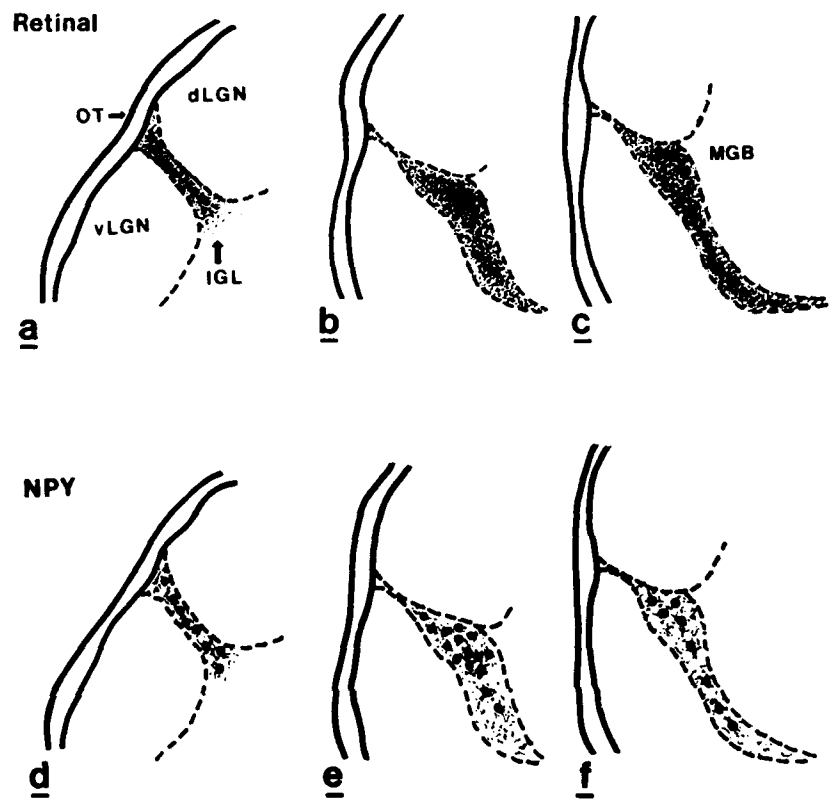

FIGURE 3. Diagrams showing the distribution of retinal afferents (**a–c**) and NPY-immunoreactive cells and axons (**d–f**) in the intergeniculate leaflet (IGL). a–c and d–f are, respectively, diagrams from rostral, mid- and caudal parts of the IGL. Abbreviations: dLGN = dorsal lateral geniculate; vLGN = ventral lateral geniculate; OT = optic tract; MGB = medial geniculate body.

become contiguous with the lateral zona incerta. At its most caudal levels, the IGL is a thin lamina of cells ventral to the medial geniculate. The distribution of retinal afferents and NPY neurons in the IGL is shown in FIGURE 3. In addition to the NPY neurons in the IGL, there is an extensive plexus of NPY- immunoreactive axons (FIG. 4). Since there are very few commissural NPY projections,[42] it seems likely that this plexus is composed of recurrent collaterals of NPY neurons projecting to the SCN.

Connections of the IGL

The principal afferent input to the IGL is from the retina. There have not been direct studies of afferents from other sources but it does appear that there are norepinephrine-containing projections from the locus coeruleus,[43] serotonin-containing projections from the midbrain raphe nuclei,[41] a substance P-containing projection from an unidentified source, perhaps the raphe nuclei,[41] a GABA-containing projection from an unidentified source,[44] a projection from the SCN and adjacent hypothalamus,[35,45,42] recurrent collat-

erals from local NPY neurons (FIG. 4) and an ENK-containing projection from the contralateral IGL.[42]

There appear to be only two efferent projections of the IGL, projections to the SCN and commissural projections to the contralateral IGL.[42] In their initial paper, Card and Moore[40] described NPY neurons in the IGL using an antiserum to avian pancreatic polypeptide which subsequently was shown to recognize the carboxy terminus of NPY.[46] They also demonstrated that bilateral lesions of the IGL produced a complete loss of NPY immunoreactivity in the SCN.[40] Unilateral lesions of the IGL produced a partial loss of NPY immunoreactivity in the SCN both ipsilateral and contralateral to the lesion, confirming the conclusion of Swanson *et al.*[37] that the geniculate-SCN projection is bilateral. These observations have been confirmed subsequently in the hamster.[47–50] Indeed, an NPY plexus has been shown in the SCN in a number of mammals indicating that this projection from the IGL to the SCN, the geniculohypothalamic tract (GHT), is a standard feature of the mammalian brain.[51,52,42] In the rat SCN, NPY-immunoreactive fibers are present in the ventral and lateral part of the nucleus from its rostral border to the caudal end of the nucleus extending to the retrochiasmatic area. The fibers in the SCN are smaller than those arising from NPY neurons in the adjacent hypothalamus and produce a very dense plexus, with numerous varicosities, largely overlapping the RHT distribution. Thus, the RHT and GHT appear likely to innervate the same population of SCN neurons.

In a recent study, Card and Moore[42] conducted an extensive analysis of lateral geniculate hypothalamic connections in the rat. Using a combination of retrograde transport

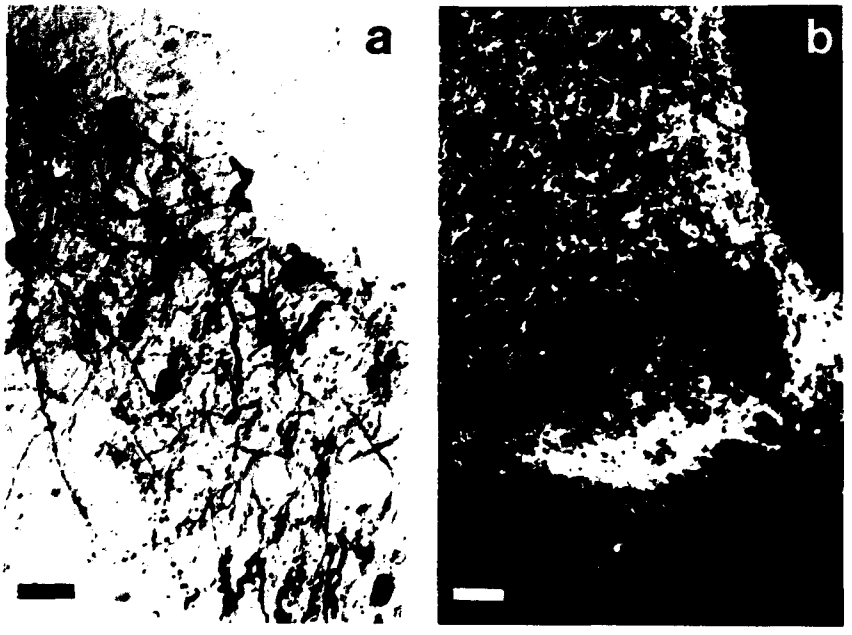

FIGURE 4. Photomicrograph showing the distribution of NPY-immunoreactive cells and fibers in IGL **(a)** (marker bar = 25 μm) and fibers in the SCN **(b)** (marker bar = 75 μm). The plexus in the ventral SCN is very dense. In addition, the SCN is surrounded by a dense plexus of NPY-immunoreactive fibers that are not of IGL origin. (From Card and Moore.[40] Reprinted by permission from the *Journal of Comparative Neurology*.)

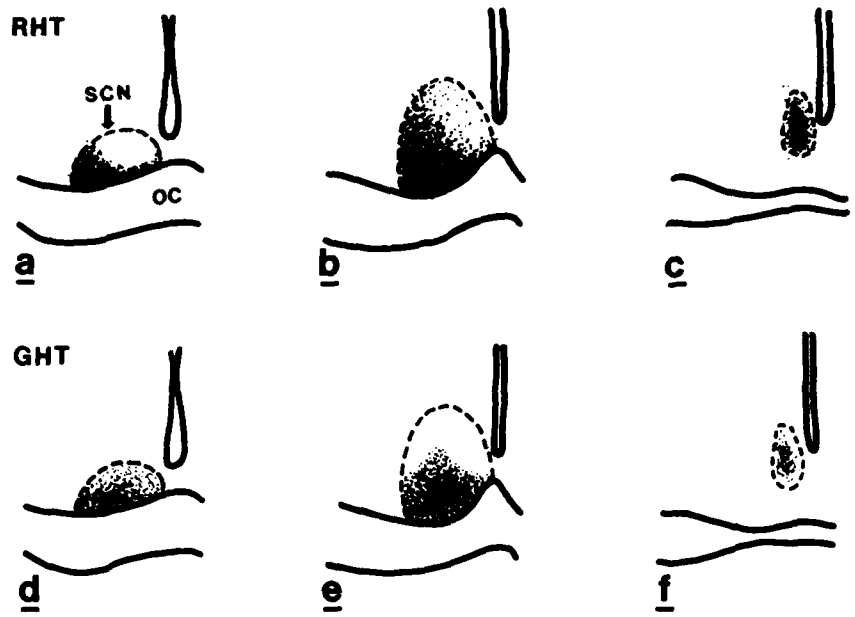

FIGURE 5. Diagrams showing the distribution of retinal afferents (**a–c**) and NPY-immunoreactive axons (**d–f**) in the suprachiasmatic nucleus (SCN). a–c and d–f are, respectively, diagrams from rostral, mid- and caudal parts of the SCN. Abbreviation: OC = optic chiasm. The retinal and NPY plexuses largely overlap but the retinal plexus is somewhat more extensive and goes beyond the borders of the nucleus.

and immunocytochemistry, they demonstrated that the NPY neurons of the IGL project nearly exclusively to the SCN. The pattern of this projection is essentially to overlap the zone of RHT afferents (FIG. 5) in the ventrolateral SCN. In ultrastructural analysis, the NPY-containing axon terminals have a lucent cytoplasm with pleomorphic vesicles including large, dense core vesicles.[53] They appear to terminate predominantly on small dendritic profiles. In addition to the NPY neurons, there is a second population of IGL neurons, one without an identified transmitter, that also projects to the SCN.[42] This group is at least as large as the NPY group. It is not known at present whether the group that does not contain NPY is functionally distinct. Lastly, there is a third IGL neuron group, ENK-containing neurons, that projects exclusively in the rat to the contralateral IGL.[42] In the hamster, these neurons project both to the SCN and the contralateral IGL.[54] The organization of RHT and GHT projections in the rat is shown schematically in FIGURE 6.

NPY in the Human CTS

There is relatively little information available on the organization of the human CTS. The SCN is a distinct feature of the anterior hypothalamus in the human brain,[55] which first appears in very rostral hypothalamus above the rostral chiasm and extends caudally above the chiasm in a periventricular location. It ends at the caudal border of the chiasm.

As in other mammals, the human SCN contains a large population of vasopressin neurons that extend from the supraoptic nucleus laterally to the paraventricular nucleus dorsally.[56]

The human lateral geniculate complex is similar in organization to that of other primates.[36] The predominant feature of the complex is a large, laminated DLG. Surrounding this is a pregeniculate nucleus with two evident subdivisions. Cells located lateral and dorsal to the DLG are relatively large and continuous with cells in the reticular nucleus of the thalamus. None of the cells in this region is NPY-immunoreactive. In contrast, there is a relatively large, wedge-shaped component of the pregeniculate nucleus that lies medial and dorsal to the DLG. This contains numerous NPY neurons and an extensive plexus of axons,[52] and seems likely to be, at least in part, the primate homologue of the IGL. However, we are not able to establish at this time whether the IGL is a component of this portion of the pregeniculate nucleus, located elsewhere, or not present in the human lateral geniculate. Part of the difficulty in establishing the location of the IGL is that we do not know the pattern of retinal afferent termination in the pregeniculate region.

Another problem arises from the pattern of NPY-immunoreactivity in the human SCN. Unlike the monkey in which there is a plexus of axons and terminals overlying the region of RHT termination,[52] the human SCN contains a large number of NPY neurons which are in the center of the nucleus, presumably overlapping the zone of RHT termination.[52] The neurons are evenly scattered over this region of the SCN (FIG. 7). There is also a plexus of very fine axons in this area. Whether this plexus arises from the local NPY neurons or reflects a GHT, as in other mammals, remains to be determined. It is of interest that the human is the only brain in which NPY neurons have been observed in the SCN. Since these neurons are present in the region of SCN where the RHT should end, it raises the possibility that there is a direct pathway in the human brain, with both NPY cell bodies and their projections located within the SCN, that by-passes the geniculate.

GHT Function

There are three general approaches that have been used to study GHT function. The first is a cellular approach using single unit recordings. It is well-known that the CTS responds to changes in luminous flux rather than to specific features of the visual environment.[57] Similarly, single units in both the SCN and IGL typically show sustained activation in response to retinal illumination, and the level of activation is generally a function of the intensity of illumination.[57,58] This is true of both IGL neurons projecting to the SCN and ones projecting to the contralateral IGL.[59] With recording of SCN neurons in hypothalamic slices *in vitro*, NPY administration has been reported to produce either excitation[60] or an initial excitation followed by a prolonged inhibition.[61] Prolonged administration of NPY produces an overall decrease in firing rate during subjective day but

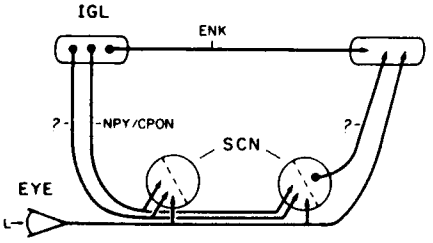

FIGURE 6. Diagram showing the organization of retinohypothalamic, geniculohypothalamic and geniculogeniculate connections in the rat. See text for description. (From Card and Moore.[42] Reprinted by permission from the *Journal of Comparative Neurology*.)

has no effect during subjective night.[61] These observations are consistent with the view that the GHT would function in entrainment.

The second approach to analysis of GHT function has been to study the effects of ablation of the IGL on circadian function. It is clear that ablation of the lateral geniculate complex, including the IGL, does not disrupt the stable entrainment of circadian rhythms.[62,10] An early study with lesions of the primary optic tracts, and presumably the GHT, reported some alteration of responses to a change in phase and a lengthened circadian period.[63] Subsequent studies, using either electrolytic or neurotoxic lesions of the IGL, have failed to find an alteration of circadian period but have routinely demonstrated changes in re-entrainment.[49,64,50] Two studies[50,65] have shown an alteration of the phase angle of entrainment in stable, entrained activity rhythms. Finally, Harrington and Rusak[66] studied the effects of IGL lesions on free-running rhythms in constant light and observed a shortened free-running period, a decrease in the likelihood of splitting and an elimination of splitting in some animals. These studies, taken together, provide further support for the view that the GHT participates in the entrainment.

The third approach has been to analyze the effects of NPY administration to the SCN on circadian function both *in vivo* and *in vitro*. The initial study of this type used avian pancreatic polypeptide,[67] or NPY,[68] superfused into the SCN region in intact, behaving animals in constant conditions. Both peptides produced dramatic, and virtually identical, changes in the phase of the circadian activity rhythm with a phase response curve (PRC) that is quite distinctive and most closely resembles the PRC obtained from administering dark pulses to animals in constant light.[69,70] This PRC is characterized by phase delays in early subjective day followed by phase advances in mid- and late subjective day with a transition to phase delays in subjective night (FIG. 8). Although the designation is certainly tentative, we shall refer to this as a D, or dark, type PRC to contrast it with the usual PRC obtained from exposing animals in constant darkness to light pulses. That PRC is typically characterized by little alteration of phase during subjective day, phase delays during early subjective night and phase advances during late subjective night.[2]

Very similar changes in phase can be obtained from acute stimulation of the IGL *in vivo*. In our study examining the effects of IGL ablation on circadian function,[11] we used

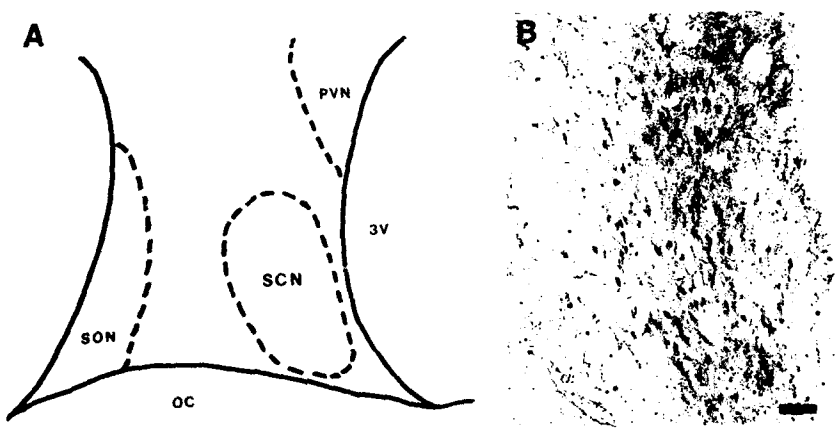

FIGURE 7. The human suprachiasmatic nucleus. **(A)** Diagram showing the location of the SCN in the chiasmal hypothalamus. Abbreviations: OC = optic chiasm; PVN = paraventricular nucleus; SCN = suprachiasmatic nucleus; SON = supraoptic nucleus; 3V = third ventricle. **(B)** Photomicrograph of NPY-immunoreactive neurons and axons in the SCN (marker bar = 100 μm).

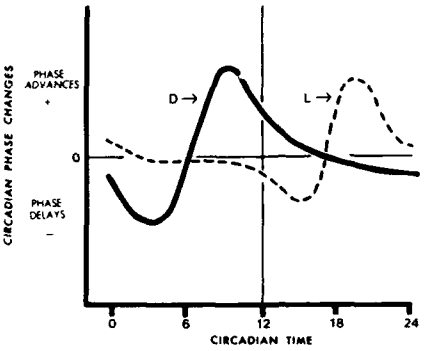

FIGURE 8. Diagram showing simulated phase response curves (PRC). See text for description. L refers to a light-type PRC and D to a dark-, or NPY-type PRC.

the excitatory neurotoxin, N-methylaspartic acid, to produce the lesions. Like other excitatory agents, this produces prolonged and excessive neuronal excitation resulting in cell death. In so doing, it will produce neuronal firing and release of transmitter from the terminals of affected neurons. The lesions in our study were made at different times of day and the animals were returned to their running wheels soon after surgery. The activity rhythms in operated animals exhibited phase changes that were dependent on the time the lesions were made and consistent with those observed by Albers and his colleagues[67,68] with NPY infusion.

Similarly, we examined acute phase changes in firing rates of single SCN neurons recorded in hypothalamic slices *in vitro*.[71] Stimulation of the optic nerve, or chiasm, produces changes in firing rate with a PRC very similar to that for light. In contrast, NPY administration produces changes in firing rate with a PRC very similar to that obtained with NPY administration *in vivo*.[68] It is of interest that the changes in firing rate occur immediately. This suggests that the change in phase of pacemaker function may also be immediate. These changes in firing rate have been maintained for three days in similar circumstances[72] indicating that they are likely to reflect a permanent change in pacemaker phase.

A series of recent studies have suggested that these two types of PRCs reflect two forms of SCN response to environmental stimulation. In the intact animal, systemic protein synthesis inhibition,[73] injection of triazolam,[74] social interaction,[75] or local injection of a protein synthesis inhibitor[76] or glutamate[77] into the SCN area all produce phase changes that resemble NPY, or dark. Similarly, administration of a protein synthesis inhibitor, calmodulin inhibitors[78] and cAMP[72] to hypothalamic slices *in vitro* produces changes in the firing rate rhythm that are similar to those produced by NPY. In contrast, pentobarbital, which produces electrical silence during administration, does not alter the firing rate rhythm but depolarization of the slice with potassium produces changes that are similar to optic chiasm stimulation.[78] These observations argue both that the regulation of circadian phase is complex and that there appear to be two principal intracellular pathways for affecting pacemaker mechanisms to change phase. One is the pathway activated by light and the other is the pathway activated by NPY. The mechanisms by which these interact to control pacemaker function in the intact animal will be a fascinating subject for future investigation.

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Role of Neuropeptide Y in Reproductive Function[a]

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INTRODUCTION

Since its discovery in 1983, many investigators have examined the distribution and activity of neuropeptide Y (NPY) in the reproductive neuroendocrine axis (see REFS. 1–3 for reviews). NPY is present throughout the hypothalamus,[4–7] pituitary,[8] and ovary,[9] and is found in high concentrations in the hypothalamo-hypophysial portal plasma.[10–12] Considerable evidence accumulated in recent years indicates that NPY modulates the secretion of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus and also affects secretion of reproductive hormones from the anterior pituitary gland.[13–21] These modulatory effects of NPY may have physiological significance in the control of the neuroendocrine events preceding ovulation. This paper will present results of studies by the author and his collaborators during the last several years elucidating the distribution and function of NPY in the reproductive neuroendocrine axis. These data are discussed in relationship to other research in the field.

Distribution of NPY

NPY immunoreactivity has been found throughout the reproductive axis of several species using both immunohistochemical and radioimmunoassay techniques (see reviews[1,3] for detailed account). Standard immunohistochemical techniques have been employed to investigate the distribution of NPY. These techniques have been described in detail and the reader is referred to the references cited for additional information. The hypothalamus displays high densities of NPY-immunoreactivity (IMR) in several nuclei which have been implicated in the control of reproductive hormone secretion.[3,5–7] The medial preoptic nucleus and horizontal limb of the diagonal band contain numerous NPY-IMR fibers in the rat, some of which surround LHRH-IMR perikarya thereby providing an anatomical basis for some of the observed functional interactions between these peptide systems.[22–24] High concentrations of NPY containing fibers are also found in the arcuate, paraventricular, dorsomedial, and periventricular nuclei, in the lateral hypothalamic area and in the median eminence. The arcuate nucleus of the rhesus monkey contains numerous NPY-IMR perikarya which appear to project axons to the infundibular stalk (FIG. 1).[8] Numerous NPY-IMR axons in the superior aspect of the infundibular stalk surround vessels of the hypothalamo-hypophysial portal system while others continue inferiorly to the neural lobe (FIG. 2). Thus, NPY is probably secreted into the portal

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FIGURE 1. Distribution of NPY-immunoreactivity in the infundibular nucleus (IN) and upper infundibular stem (UIS) of the rhesus monkey. High concentration of NPY-labeled perikarya in the ventral IN. Note the stained fibers and perikarya in the UIS (*arrows*), some near blood vessels. Bar = 100 μm × 106. (McDonald, J. K., J. Tigges, M. Tigges & C. Reich, unpublished observations.)

vasculature of rhesus monkeys and other nonhuman primates and bathes the anterior pituitary gland. In the rat, NPY-IMR fibers surround capillaries in the median eminence although the density is lower than in the rhesus monkey (FIG. 3).[10] Ultrastructural immunohistochemical analysis of the rat median eminence shows NPY-IMR axons adjacent to the perivascular space near fenestrated capillaries (Calka & McDonald, unpublished observations). High levels of NPY have been measured in the portal plasma of male and female rats (discussed below). In view of the direct effects of NPY on pituitary hormone secretion, these anatomical observations in the rhesus suggest that NPY may directly influence anterior pituitary function in primates.

The ovary is richly innervated with NPY-containing fibers which are distributed primarily around the vasculature although other fibers course through the interstitial tissue and some are closely associated with follicles (FIG. 4).[9] A vasomotor function of NPY in some perivascular ovarian nerves is likely although the function of interstitial and perifollicular NPY-containing fibers remains unknown. Preliminary studies suggest that NPY alone or together with follicle-stimulating hormone (FSH) is a poor stimulus for estradiol or progesterone secretion from granulosa cell cultures. A potential endocrine role in the ovary, perhaps to affect secretion of some nonsteroidal substance remains to be determined.

FIGURE 2. Relationship of NPY-immunoreactive fibers to portal vessels within the middle (M) IS and adjacent to the anterior pituitary (AP) of the rhesus monkey. **(A)** Dense accumulation of NPY-positive varicosities surrounding several small and large vessels in the MIS. The labeled vessel (v) distributes within the AP. Bar = 50 μm. ×169. **(B)** Many varicose NPY-containing fibers surround vessels (v) leading directly into the dorsal AP. Other fibers continue down the IS. Bar = 50 μm. ×106. Abbreviations as in legend to FIGURE 1. (FIG. 2A from McDonald *et al.*[8] Reprinted by permission from *Cell and Tissue Research.*)

NPY Modulation of LHRH Secretion from the Hypothalamus

Effects of Cerebroventricular Injection of NPY on LH Release

In 1981, during the course of immunocytochemical studies of pancreatic polypeptide (PP) immunoreactivity in the developing visual cortex,[25] I became interested in the very high levels of NPY-IMR in the arcuate, periventricular and medial preoptic nuclei of the hypothalamus. We investigated a potential neuroendocrine role for PP by injecting synthetic avian (a)PP and bovine (b)PP into the third cerebral ventricle of conscious, unrestrained ovariectomized rats fitted with an indwelling intraatrial cannula. In 1983 we reported that both peptides significantly decreased plasma levels of luteinizing hormone (LH) in a dose-related manner.[26,27] FSH levels were not affected. NPY was discovered during the course of these studies and it soon became apparent to investigators working in this field that previous reports of PP-like immunoreactivity in the brain were due to cross-reactivity of the PP antisera with the endogenous peptide NPY.[28] We immediately tested NPY *in vivo* and found that it also decreased plasma levels of LH in ovariectomized rats in a dose-dependent manner and was effective at doses as low as 20 ng (4.7 pmol) (FIG. 5). We hypothesized that these inhibitory effects of NPY on LH release were due to suppression of LHRH secretion from the hypothalamus.[13,29]

This hypothesis was tested subsequently by examining the ability of NPY to inhibit the pulsatile release of LHRH which drives episodic LH secretion in ovariectomized rats.[15] Once again, NPY was injected into the third cerebral ventricle of conscious freely-moving rats with indwelling intraatrial cannulae. Blood was taken every 10 minutes for three hours. NPY treatment suppressed the pulse frequency and amplitude of LH secretion for several hours in rats receiving 5 μg (FIGS. 6,7). Administration of 0.5 μg of NPY also significantly decreased pulsatile LH release during the next 50 minutes with signs of recovery soon thereafter (FIG. 6). These suppressive effects of NPY were not due to a decreased secretory capacity of gonadotrophs since administration of LHRH (10 ng/100 g BW) i.v. at the time of maximal NPY suppression of LH release caused a 1239%

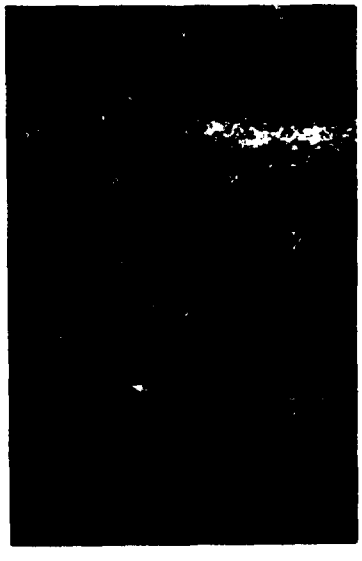

FIGURE 3. Distribution of NPY-immunoreactive fibers in the subependymal zone, internal zone (ZI), and external zone (ZE) of the rat median eminence. The location of the third ventricle (v) is indicated. The *arrow* is located in a portal vessel and points to an adjacent NPY-labeled fiber. ×256. (From McDonald *et al.*[10] Reprinted by permission from *Neuroendocrinology*.)

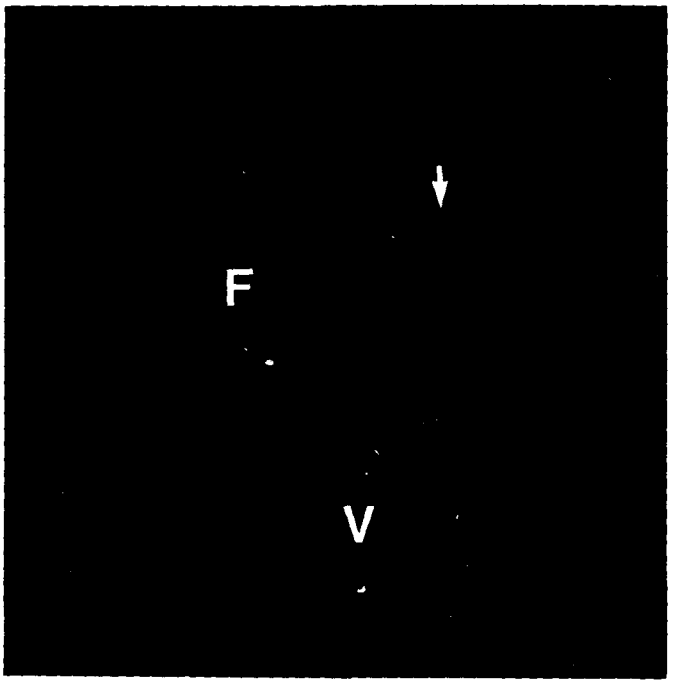

FIGURE 4. NPY- immunofluorescent fibers in the rat ovary which surround this vessel (V) in longitudinal section may give rise to fibers which appear intimately associated with a follicle (F). ×256. (From McDonald *et al.*[9] Reprinted by permission from *Endocrinology*.)

increase in plasma LH levels compared to a 124% increase in control animals injected with saline into the third ventricle (FIG. 8). These data support the notion that NPY may be a component of the LHRH pulse generating system. Perhaps the NPY-containing neurons of the arcuate nucleus exert a modulatory influence on the pulse generator in the basal hypothalamus.[15] Other investigators have observed that push-pull perfusion of NPY into the hypothalamus of ovariectomized rabbits decreases pulsatile LHRH release.[19]

Estrogen changes the direction of the effect of NPY on LH and LHRH secretion. I.c.v. injection of NPY in ovariectomized rats treated with estrogen and progesterone produces a transient stimulation of LH release.[14] Gonadally intact rabbits also display NPY-induced LHRH secretion during push-pull perfusion of the hypothalamus.[19] Investigations carried out *in vitro* suggest that the stimulatory effects of NPY on LHRH secretion in steroid-treated ovariectomized rats may occur in the medial basal hypothalamus,[17] perhaps specifically at the median eminence.[16] In contrast, NPY suppression of LHRH secretion in ovariectomized rats seems to require additional hypothalamic nuclei. We initiated an extensive series of studies to explore the steroid dependency of NPY modulation of LHRH release from the median eminence *in vitro*.

NPY Modulation of LHRH Secretion In Vitro

Role of Estrogen. Rats were bilaterally ovariectomized and 2–4 weeks later received one or several Silastic tubes s.c. containing estradiol benzoate in oil or oil vehicle. Three

days later, animals were sacrificed, trunk blood collected and the median eminence removed as described in detail and placed individually in test tubes (see REF. 16 for experimental details). After several rinses with modified Krebs-Ringer bicarbonate medium containing glucose, a 30-minute basal sample was taken followed by administration of NPY (0.1–10 μM) during a 30-minute test period. Medium was assayed for LHRH. NPY had no effect on LHRH secretion from median eminences obtained from ovariectomized rats and did not significantly modify potassium-induced LHRH release. Thus, NPY suppression of LH release *in vivo* may be mediated by other sites than the median eminence. The estrogen treatments produced physiological plasma levels of estradiol similar to those measured during the estrous cycle.[16] Median eminences were harvested in the morning between 0900–1000 h when plasma levels of LH were low, significantly before the afternoon LH surge. However, the median eminence content of LHRH and the basal release of LHRH were significantly increased by these treatments. NPY levels were significantly increased only following treatment with the highest dose of estrogen. Therefore all data concerning LHRH release during the test period were expressed in terms of the basal release of LHRH in the preceding period. When plasma estradiol levels were low (17 pg/ml), as in diestrous, NPY produced a dose-related increase in LHRH release. Treatment with two or four times this dose of estrogen produced higher physiological levels of circulating estradiol (30 and 73 pg/ml) comparable to levels on proestrous. These higher doses of estrogen also caused a dose-related increase in the sensitivity of the median eminence to NPY. NPY-stimulated LHRH secretion increased significantly in animals with higher plasma levels of estradiol (FIG. 9). Thus it seems that the median

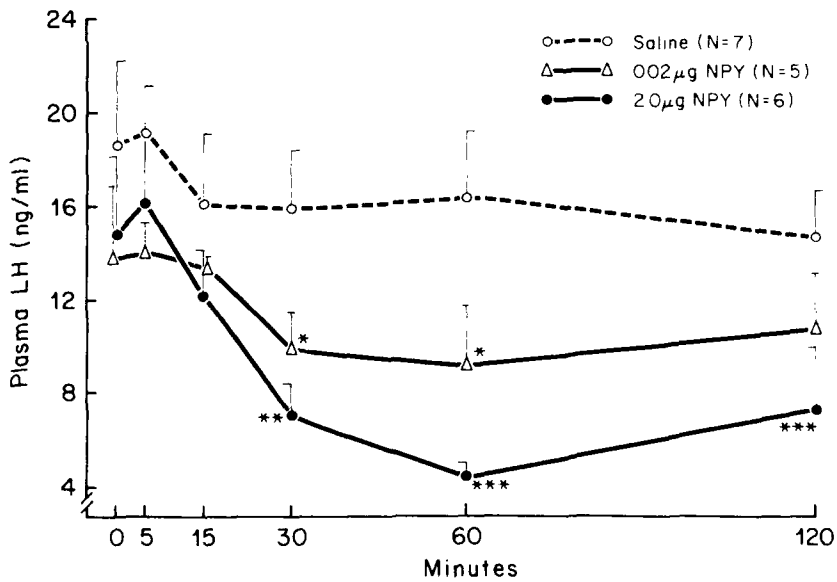

FIGURE 5. Effect of the third cerebroventricular injection (3V) of 0.9% saline and NPY on plasma levels of LH in conscious, unrestrained, ovariectomized rats. In this figure, *points* and *vertical bars* represent mean values ± SEM. *Symbols adjacent to points* represent the level of significance when compared to saline-injected controls. *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.005$. NPY doses were 2.0 and 0.02 μg. (From McDonald *et al.*[15] Reprinted by permission from the National Academy of Sciences.)

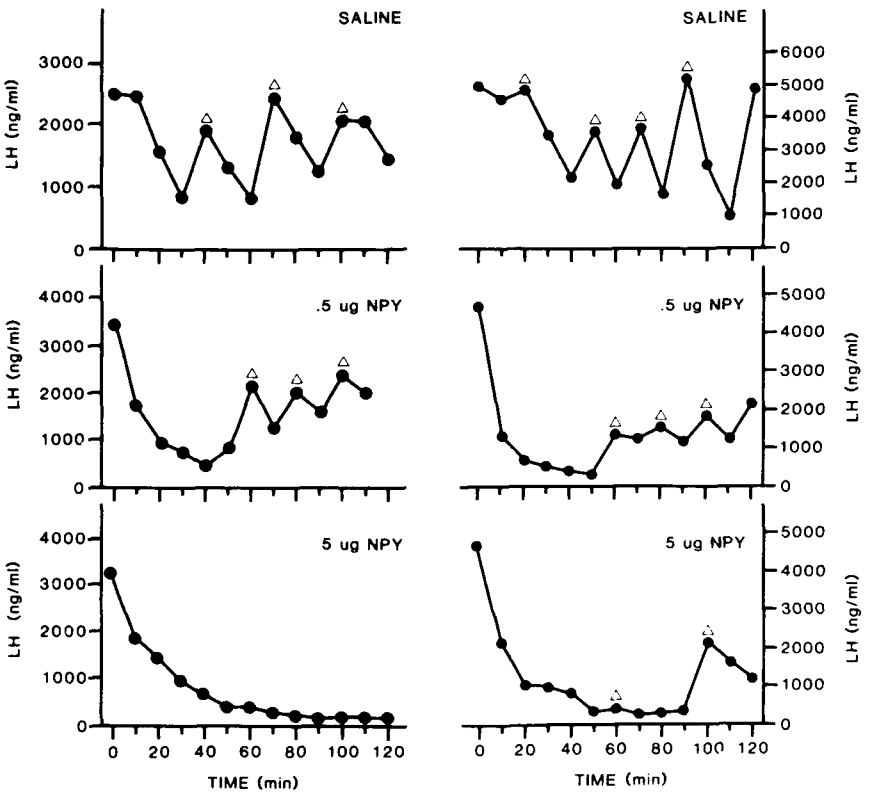

FIGURE 6. Individual profiles of LH secretion (nanograms per ml) in OVX rats receiving a 3V-injection of NPY (5.0 or 0.5 μg/2 μl) or saline (2 μl) at 0 min. *Symbol* indicates a pulse of LH secretion. (From McDonald *et al.*[15] Reprinted by permission from *Endocrinology*.)

eminence is sufficient to demonstrate NPY stimulation of LHRH release in estrogen-treated ovariectomized rats.

Effects of Progesterone in Estrogen-Treated Ovariectomized Rats: In view of the ability of physiological plasma levels of estrogen to change the direction of the effect of NPY from suppression in ovariectomized rats to stimulation, and the reported enhancement by progesterone of NPY-stimulated LHRH release from medial basal hypothalamic fragments *in vitro*,[17] we performed experiments to investigate the hypothesis that progesterone increases NPY-stimulated LHRH secretion from the median eminence *in vitro*.[30] Rats were bilaterally ovariectomized and implanted s.c. with Silastic capsules containing estradiol benzoate in oil (235 μg/ml) to produce physiological plasma levels of estradiol, as described above. Three days later, animals were injected between 0800–0830 hours with progesterone (1, 2, or 19 mg s.c. per animal) or oil vehicle. Animals were sacrificed three hours after injection and trunk blood was collected. Median eminences were removed and either sonicated in 0.1N HCl to determine levels of NPY and LHRH, or placed in test tubes containing culture medium to examine the effects of NPY on LHRH secretion. The 1- and 2-mg doses of progesterone produced physiological plasma levels of progesterone (30 and 64 ng/ml, respectively) while injection of 19 mg

resulted in pharmacological levels (534 ng/ml) that were 10 times greater than during the estrous cycle. In no case did progesterone treatment enhance NPY-stimulated LHRH secretion from the median eminence *in vitro*. Injection of 1 mg had no effect on the median eminence content of LHRH, while 2 mg and 19 mg of progesterone significantly increased LHRH levels. In spite of these changes, NPY stimulation of LHRH release was slightly less effective in rats treated with the 2-mg dose, and was significantly reduced in animals receiving 19 mg of progesterone, although these animals displayed significantly elevated basal release of LHRH.[30] These results indicate that progesterone administration to ovariectomized estrogen-treated rats does not enhance NPY-stimulated LHRH release from the median eminence when examined three hours later. LHRH levels are increasing at this time, well before the afternoon LH surge, yet NPY is apparently less effective in stimulating the release of available LHRH. It is interesting to speculate that progesterone

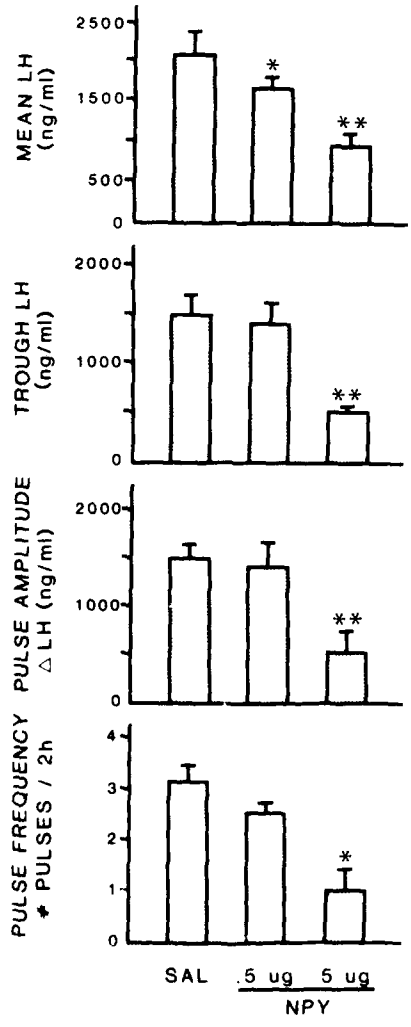

FIGURE 7. This four-panel histogram summarizes the effects of NPY (5.0 or 0.5 μg) on the mean level, trough level, pulse amplitude, and pulse frequency of LH secretion over the 2-h sampling period (mean ± SEM). *, $p < 0.05$; **, $p < 0.01$ (vs saline (SAL)-injected controls). (From McDonald *et al.*[15] Reprinted by permission from *Endocrinology*.)

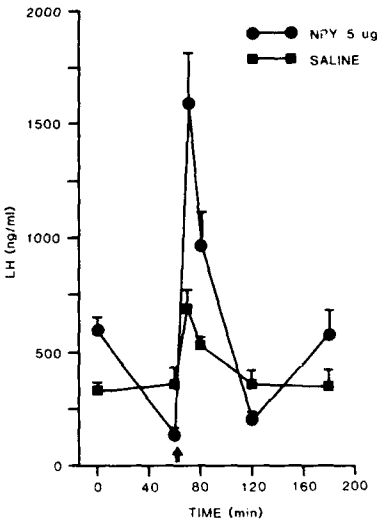

FIGURE 8. Effect of NPY on the pituitary LH response to iv LHRH. Plasma levels of LH (mean ± SEM) in OVX rats receiving a 3V injection of NPY (5 μg: *circle*) or saline (*square*). At 60 min, when LH levels were significantly reduced in NPY-treated rats, LHRH (10 ng/100 g BW) was injected iv (*arrow*). Saline injected animals showed a 124% increase in plasma LH 10 min after LHRH administration. In contrast, NPY-treated animals responded with a 1239% increase in LH release ($p < 0.01$ vs controls). (From McDonald *et al.*[15] Reprinted by permission from *Endocrinology*.).

exerts an inhibitory influence during and after the afternoon LH surge to limit NPY stimulation of LHRH secretion.[30] Additional research employing both *in vitro* and *in vivo* methods is needed to fully explore the effects of progesterone on NPY-induced LHRH secretion throughout the afternoon and evening of the LH surge. Taken together, these

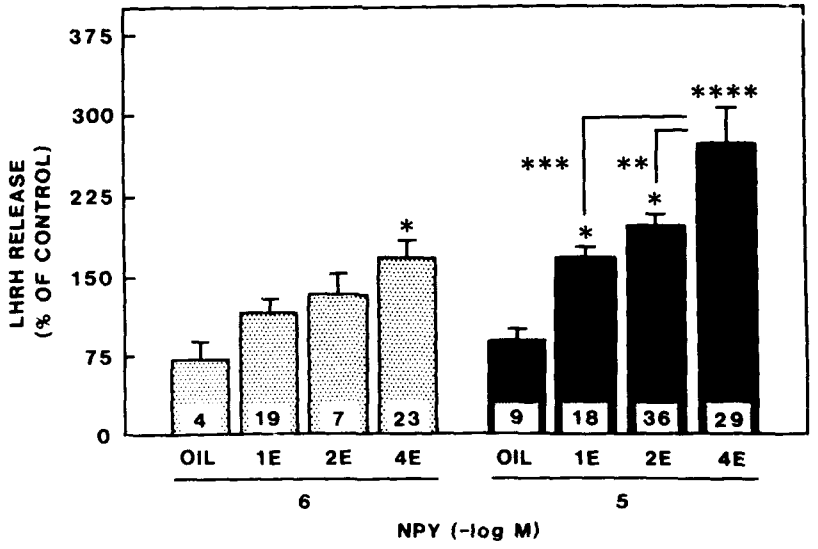

FIGURE 9. Effects of estrogen (1E = 1 10-mm implant; 2E = 2 10-mm implants; 4E = 2 20-mm implants of estradiol benzoate 235 μg/ml) or vehicle treatment on NPY (10^{-6} and 10^{-5} M)-stimulated LHRH release from ME fragments of OVX rats. ****, $p < 0.001$, 4E vs OIL for 10^{-5} M NPY; ***, $p < 0.005$, 4E vs 1E for 10^{-5} M NPY; **, $p < 0.01$, 4E vs 2E for 10^{-5} M NPY; *, $p < 0.05$, 2E and 1E vs OIL for 10^{-5} M NPY, and 4E vs OIL for 10^{-6} M NPY. (From Sabatino *et al.*[16] Reprinted by permission from *Endocrinology*.)

studies indicate that estrogen exerts a major influence to sensitize the median eminence to NPY. The specific physiological role of progesterone, if any, remains to be elucidated.

Role of Calcium: We also explored the role of extracellular calcium (Ca^{2+}) in NPY-stimulated LHRH release from the median eminence since influx of calcium often accompanies changes in hormone release. As expected, decreasing the extracellular Ca^{2+} concentration significantly inhibited the stimulatory effect of 56 mM potassium. However, lowering the Ca^{2+} concentration in the incubation medium, or eliminating Ca^{2+} altogether and adding EGTA had no effect on NPY-stimulated LHRH release. In fact the stimulatory effects of NPY were maintained in Ca^{2+}-free medium with EGTA and 2.5 mM cobalt (FIG. 10). Movement of intracellular Ca^{2+}, however, is a necessary step in

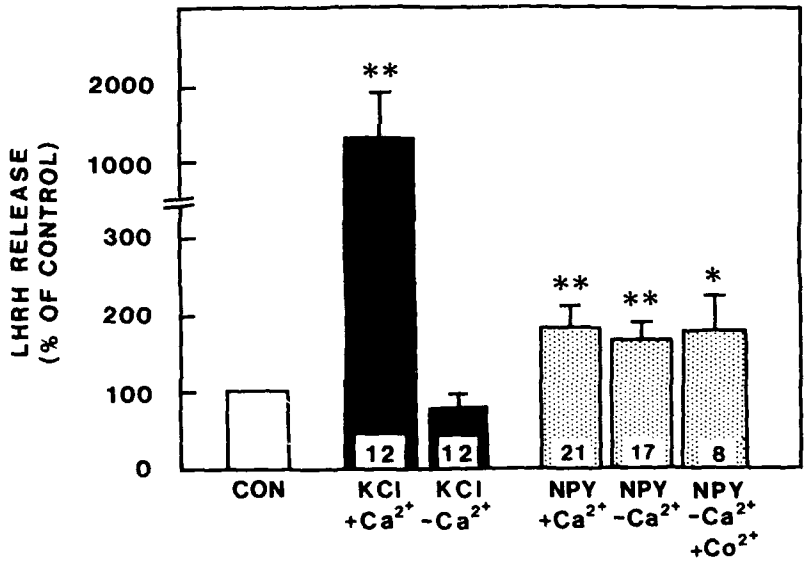

FIGURE 10. Effect of Ca^{2+}-free medium on NPY (10^{-5} M)- and KCl (56 mM)-stimulated LHRH release from ME fragments obtained from 2E-treated OVX rats. Ca^{2+}-free medium is designated $-Ca^{2+}$ whereas normal medium containing 2.5 mM Ca^{2+} is designated $+Ca^{2+}$. **, $p < 0.001$, KCl ($+Ca^{2+}$) vs KCl ($-Ca^{2+}$) or control (CON) period and NPY ($+Ca^{2+}$ or $-Ca^{2+}$) vs control period; *, $p < 0.05$, NPY ($-Ca^{2+}$ with Co^{2+} (2.5 mM)) vs control period. There were no significant differences between NPY groups. (From Sabatino *et al.*[16] Reprinted by permission from *Endocrinology.*)

NPY-induced LHRH secretion. Addition of 3,4,5-trimethoxybenzoic acid 8-(dimethylamino) octyl ester (TMB-8) at 50 μM, which blocks intracellular Ca^{2+} translocation, significantly reduced NPY (10 μM)-stimulated LHRH release from median eminence fragments from ovariectomized rats treated with 2 20 mm Silastic implants containing estradiol benzoate in oil (235 μg/ml) (Sabatino & McDonald, unpublished observations). Similar results have been reported recently in abstract form.[31]

Secretion of NPY into the Hypothalamo-Hypophysial Portal Circulation

Results from immunohistochemical studies of PP and later NPY, showed labelled fibers in the median eminence. Most of these fibers located in the internal layer are

destined for the neural lobe and respond to osmotic stimuli.[32] Other stained fibers are located in the external layer and lateral aspect of the median eminence in the vicinity of capillaries of the hypothalamo-hypophysial portal system (FIG. 3). These observations combined with the direct stimulatory effect of NPY on hormone secretion from anterior pituitary cells lead us to hypothesize that NPY is released from fibers in the median eminence into the perivascular space surrounding these fenestrated capillaries. This hypothesis was investigated by collecting portal blood from the transected pituitary stalk of male rats and comparing the levels of NPY-IMR with those in a sample of peripheral blood taken simultaneously.[10] Increasing volumes of plasma produced parallel displacement of labelled NPY in the RIA. In addition, chromatographic characterization of these samples with successive HPLC runs to obtain high resolution of NPY-IMR showed that this IMR eluted as a single peak which displayed a slightly shorter retention time than the porcine NPY standard. This difference was likely due to the methionine for leucine substitution which occurs in position 17 in rat NPY versus porcine NPY. Human and rat NPY elute slightly earlier than porcine NPY in these HPLC solvent and gradient conditions. Portal plasma levels of NPY were 3–4 times greater than in the peripheral circulation. These results support the hypothesis that NPY is released into the portal circulation and acquire additional significance in view of NPY stimulation of anterior pituitary hormone secretion. Other investigators have provided compelling evidence that NPY levels in the portal plasma are elevated in concert with LHRH before the LH surge in estrogen- and progesterone-treated ovariectomized rats, and also before the first LH surge at pubertal onset.[11,12] These results combined with those in the next section strongly suggest that NPY exerts a direct regulatory influence on anterior pituitary function.

NPY Modulation of Reproductive Hormone Secretion from the Anterior Pituitary Gland

In 1984, we reported that application of NPY stimulated the release of LH, FSH and growth hormone from dispersed anterior pituitary cells which were obtained from ovariectomized rats, mixed with Biogel P2, and perifused in syringe columns (FIG. 11).[13,29] These original findings of direct effects of NPY on anterior pituitary hormone release were later supported by other investigators.[20] However the test system apparently has a great influence on the ability to detect NPY activity.[18,33,34] Use of hemipituitary fragments or long-term cultures of dispersed cells did not reveal any effect of NPY on hormone release.[18,34] Khorram *et al.*[20] placed quartered rabbit anterior pituitaries in a perifusion system and demonstrated transient NPY-stimulated LH and FSH secretion from pituitaries of ovariectomized animals and a sustained increase in LH and FSH release from glands of intact animals. This indicates that ovarian steroids modulate the sensitivity of the anterior pituitary gland to NPY. Other investigators have reported that NPY exerts a slight but significant stimulation of LH release and also increases the LH secretory response of gonadotrophs to LHRH using hemipituitary fragments.[18] When NPY was applied to three-day cultures of dispersed anterior pituitary cells there was no effect on LH release but the response to LHRH was potentiated.[18] Peripheral injection of NPY antiserum blocked the LH surge in ovariectomized rats treated with estrogen and progesterone. These investigators suggested that NPY serves in a modulatory role to prime gonadotrophs to LHRH.[12]

SUMMARY

NPY acts both at the hypothalamus and the anterior pituitary gland to modulate reproductive hormone secretion (FIG. 12). Within the hypothalamus, NPY stimulates

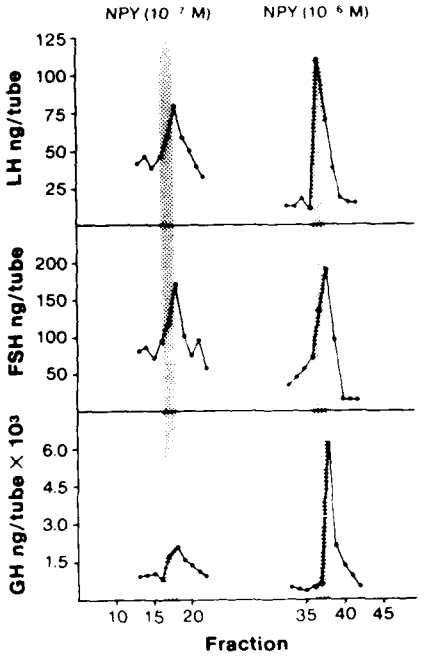

FIGURE 11. LH, FSH, and GH released from a perifused cell column (BioGel P-2, 0.4 × 1.5 cm) loaded with 7×10^6 dispersed anterior pituitary cells (ovariectomized female rat donors). Five-minute fractions were collected and NPY (10^{-7} and 10^{-6} M) was included in the medium for the 10-min exposure periods indicated by the *stippled bars*. (From McDonald *et al.*[15] Reprinted by permission from the National Academy of Sciences.)

LHRH secretion in the presence of physiological levels of estrogen and suppresses pulsatile LHRH release following ovariectomy. Intracerebroventricular injection of NPY antiserum blocks or delays the LH surge in steroid-primed ovariectomized rats,[35] thereby adding support for a physiological role of NPY in the neuroendocrine events preceding ovulation. Blockade of alpha 2 adrenergic receptors decreases NPY-stimulated LH release in steroid-primed rats implying a potential noradrenergic mediation of NPY activity.[36] Physiological levels of progesterone do not augment, and may actually suppress NPY-induced LHRH secretion *in vitro* from median eminences obtained from estrogen-primed ovariectomized rats. The physiological role of progesterone, if any, in modulating NPY effects on LHRH release remains to be determined. Little, if anything, is known about the

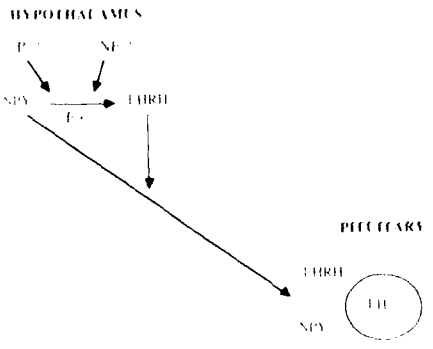

FIGURE 12. Summary diagram showing the dual sites of action of NPY in modulating reproductive hormone release from the hypothalamus and anterior pituitary gland. See Summary in text for additional discussion. E + represents the permissive effect of estrogen on NPY-stimulated LHRH release from the hypothalamus. The physiological roles of progesterone (P) and norepinephrine (NE) in modulating this influence of NPY remain to be determined. The *long oblique arrow* represents the hypothalamo-hypophysial portal vessels which transport both NPY and LHRH to the gonadotroph (*circle*) where these peptides appear to act independently and also interact in the modulation of LH secretion.

NPY receptor in the median eminence or the intracellular mechanisms which transduce the NPY signal into activation of LHRH release in estrogen-treated ovariectomized rats although translocation of intracellular calcium is required. Equally puzzling is the mechanism of desensitization of the LHRH-releasing mechanisms of the median eminence of ovariectomized rats or the specific site of NPY suppression of pulsatile LHRH secretion.

NPY is released into the hypothalamo-hypophysial portal circulation and this appears correlated with LHRH secretion before the LH surge. NPY affects LH and FSH release from anterior pituitary cells *in vitro* and enhances LHRH-induced LH secretion.

Taken together, the studies described above suggest an important physiological role for NPY as a modulator of neuroendocrine activity which culminates in the preovulatory surge of LH.

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Hypothalamic Neuropeptide Y: a Circuit in the Regulation of Gonadotropin Secretion and Feeding Behavior[a]

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INTRODUCTION

Neuropeptide Y (NPY) is an important messenger molecule in the neuroendocrine control of reproduction and appetitive behavior. The early history of the development of our idea that members of the pancreatic polypeptide family, including NPY, may be involved in the hypothalamic control of pituitary luteinizing hormone (LH) secretion, and of feeding and sexual behaviors is described elsewhere.[1] In this article the mechanism of action of NPY at cellular and subcellular levels in stimulation of LH release and feeding behavior is described. In particular, attention is focused on the site and specificity of NPY action, the functional connectivity of NPY with other neuropeptidergic and monoaminergic systems in the hypothalamus and brain stem (BS), and whether NPY is a physiological neurochemical signal in the induction of ovulation and stimulation of appetite.

ROLE OF NPY IN THE CONTROL OF HYPOTHALAMIC LHRH-PITUITARY LH SECRETION

Depending upon the existing status of the gonadal steroidal milieu, central administration of NPY either inhibits or stimulates the release of LH from the anterior pituitary.[2] However, the excitatory nature of NPY is physiologically relevant because NPY stimulates LH release in gonad-intact rats and rabbits and in ovariectomized (ovx) rats pretreated with ovarian steroids.[2-6] Further, this excitation of LH release was found to be due to NPY action at two sites, one in the hypothalamus wherein it acted to stimulate LH releasing hormone (LHRH) release, and the other at the level of the pituitary, where it potentiated the release of LH induced by LHRH.[7-9]

Mode of NPY Action in Stimulation of Hypothalamic LHRH Release

Our studies showed that an optimal level of communication between hypothalamic NPY and LHRH neurons required priming with gonadal steroids.[8,10] In general, NPY

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failed to alter the *in vitro* basal LHRH release from the medial basal hypothalamus (MBH) of steroid-unprimed ovx rats, but priming with either estradiol 17β (E_2) alone or in combination with progesterone (P) resulted in stimulation of LHRH release by NPY. In fact, NPY readily stimulated LHRH release from the MBH of rats primed with physiological levels of E_2 alone. Furthermore, NPY potentiated the release of LHRH in response to K^+-induced depolarization from the MBH of ovx rats. Therefore, it seemed that NPY can not only stimulate LHRH on its own, but may also potentiate the response of LHRH neurons to depolarization produced by those synaptic inputs, such as adrenergic neurotransmitters, which stimulate LHRH and LH release.

The anatomical evidence clearly shows that NPY exists in a number of neuronal populations in the brain stem (BS) which produce norepinephrine (NE) and epinephrine (EPI).[11-13] To what extent do these cells, showing coexistence of NPY and adrenergic transmitters, project into the hypothalamic sites innervated by LHRH neurons? What is the nature of interaction between adrenergic transmitters and NPY in stimulation of the LHRH-LH axis? Bilateral neural transection (NT) at the level of the dorsal tegmentum in the mesencephalon not only drastically decreased NE and EPI levels in the POA and MBH, but also reduced the levels of NPY in selected sites in the preoptic-tuberal pathway.[14] Severing these BS projections to the hypothalamus reduced NPY concentrations in the medial preoptic area (MPOA) and median eminence (ME). It appeared that the afferents of the two populations of NPY-producing cells, the remote BS population which also contain NE or EPI, and the local arcuate nucleus (ARC) population project into areas innervated by LHRH neurons. Based on the known topography of the LHRH network in the diencephalon, it is reasonable to suggest that stimulation of LH release by NPY occurred via axo-dendritic or axo-somatic synapses in the MPOA, and via axo-axonic synapses in the ME region.

Allen *et al.*[15] showed that such links between NPY and LHRH at the morphological level are functionally operational in the ovarian steroid-primed ovx rats. Doses of NPY and NE, that on their own were minimally effective in stimulating LH release, when administered together induced a LH response greater than the sum of the individual responses. On the other hand, combined doses of NPY and NE, which were either ineffective or maximally effective, failed to induce an additive response. This interplay between NPY and adrenergic transmitters is uniquely complex. We were unable to block the NPY-induced LH release either by selectively blocking α- or β-adrenoreceptors or dopamine receptors, or by stimulating opiate receptors in the hypothalamus. Intriguingly, however, stimulation of LH release by NPY was significantly suppressed by prior blockade of α_2-adrenoreceptors. Therefore, it appeared that either α_2-adrenoreceptors located at a postsynaptic site, mediated the excitatory LHRH response, or that it was necessary to sustain optimal postsynaptic α_2-adrenoreceptor activity by endogenous adrenergic transmitters, to fully express the action of NPY. However, currently we cannot rule out the possibility that NPY may directly modulate hypothalamic α_2-adrenoreceptor membrane binding sites that are coupled to the phosphatidylinositol second messenger system.

With respect to the receptor specificity of the NPY action, it is likely that the Y_1 receptor subtype mediates the excitatory effects of NPY on LHRH release. We observed that a specific Y_1 receptor agonist, [Leu^{31},Pro^{34}]-NPY,[16] readily stimulated LHRH, whereas the Y_2 receptor agonist, NPY_{13-36},[17,18] was completely ineffective in stimulating LHRH release from the ME of ovarian steroid-primed ovx rats.[19] Furthermore, stimulation of LHRH release by Y_1 receptor activation does not require extracellular Ca^{2+} because LHRH release by NPY persisted in Ca^{2+}-free/EGTA medium. In contrast, we observed that two antagonists of intracellular Ca^{2+} mobilization, TMB-8 and ryanodine, blocked the NPY-induced LHRH release.[19] This line of evidence favors the idea that activation of the Y_1 receptor subtype subsequent to mobilization of intracellular Ca^{2+} results in stimulation of LHRH release.[10,19]

Mode of NPY Action at the Level of the Pituitary

The morphological and experimental evidence favors a neurohumoral role of NPY in stimulation of LH release directly from the pituitary gland.[11,12,20] NPY was found to stimulate LH release *in vitro* from either hemipituitaries or dispersed pituitary cells. Further analysis revealed that the direct stimulatory effect of NPY on LH release was very weak as compared to that of the physiological releaser LHRH, and demonstration of even this marginal response was dependent upon a number of factors such as NPY pulse duration and the previous hormonal status of the donor.[7,9,10]

On the other hand, NPY interacted with LHRH in a unique fashion. We found that NPY potentiated LH release in response to LHRH.[7,9] When added to dispersed pituitary cell incubations, NPY augmented the release of LH by LHRH in a dose-related fashion. Further, although we have not yet observed direct binding of NPY to pituitary membranes, NPY enhanced LHRH binding to its receptors on pituitary membranes, an action likely to contribute to augmented LH release by LHRH.[21] Furthermore, unlike the postsynaptic LHRH stimulation response in the hypothalamus,[7,8] we observed that the dihydropyridine Ca^{2+} channel antagonist, nitrendipine, blocked the effects of NPY on LHRH-induced LH release.[10,22,23] Further, NPY enhanced the LHRH-induced increase in cytosolic Ca^{2+} as well as the number of pituitary cells displaying increased intracellular Ca^{2+} in response to LHRH.[22,23]

Consequently, we envision that NPY participates in the neuroendocrine control of LHRH-LH axis in a novel way. At the two disparate sites—hypothalamus and pituitary—NPY displays a unique cooperativity with classial adrenergic transmitters and the LHRH peptidergic system by different intracellular modes of action, the optimal manifestation of which is apparently dependent upon the gonadal steroid milieu.

Physiological Significance

Participation of NPY in the Induction of Ovulation

Because it readily stimulates both LHRH and LH release, we hypothesized that the NPY network may be an important component of the hypothalamic neural circuitry responsible for the preovulatory discharge of LH.[24] We observed parallel changes in NPY and LHRH levels in the ME in association with the afternoon LHRH and LH hypersecretion either induced by ovarian steroids in ovx rats or that occurring spontaneously on the afternoon of proestrus.[25,26] On proestrus, serum LH levels were basal between 1000 and 1300 h, rose significantly at 1500 h and plateaued between 1600 and 1800 h. Of the five hypothalamic sites examined, NPY levels displayed marked fluctuations only in the ME in close association with the LH surge.[26] NPY concentrations were low between 1000 and 1300 h, and rose abruptly at 1400 h preceding the onset of LH rise at 1500 h. These elevated levels were maintained until 1600 h during which time serum LH rose to a plateau and then fell at 1800 h to the low range seen between 1000 h and 1300 h. The fact that these dynamic changes in the ME NPY levels occur in parallel with LHRH levels on proestrus, but do not occur on diestrus II, is in line with our hypothesis that a subset of NPY neurons terminating in the ME may be a component of the excitatory input that, either independently or in co-action with the adrenergic system, is responsible for the induction of preovulatory LH release.[24] Indeed, the observations that NPY stimulated LHRH release,[8,19] passive immunization with NPY antisera blocked the LH surge,[27] and high levels of NPY were detected in the hypophyseal portal plasma in the afternoon of proestrus,[28] favor the notion that elevated ME NPY levels are associated with an increase in NPY discharge for a two-pronged involvement in the preovulatory discharge of LH.

one to increase hypothalamic LHRH release and the other to augment the pituitary response to LHRH.

The observations of parallel increments of NPY and LHRH levels in the ME posed two additional questions. What is the source of the increments in ME NPY levels? Is there a common neural mechanism that activates the diverse hypothalamic neurochemical signals implicated in the initiation of the preovulatory LH surge? Based on our studies,[24] we suspect that the abrupt increments in the ME NPY levels are pimarily due to increased processing and elaboration of NPY in the ME nerve terminals originating primarily from the ARC, and in response to a heavy local functional demand to sustain a high rate of NPY efflux required for the LH surge. With respect to the question of the neural factors that trigger these dynamic changes on proestrus, the emerging evidence has assigned a crucial role to the endogenous opioid tone (EOP) in the preoptic tuberal pathway.[29,30] Extending these findings further, we observed that a decrease in opioid tone with infusion of the opiate receptor antagonist, naloxone, in estrogen-primed ovx rats, advanced the onset and amplified the magnitude of the LH surge in the afternoon.[31] In association with this augmentation, naloxone infusion significantly increased NPY concentrations selectively in the ME, MPOA and ARC, sites previously implicated in induction of the preovulatory LH surge. Further, we observed that naloxone stimulated the *in vitro* efflux of both NPY and LHRH from the MBH.[31] Since previous studies showed that a transient decrease in opioid tone induced by the neural clock (NC) on proestrus and in steroid-treated ovx rats initiates and sustains the preovulatory LH surge,[24,29,30] the findings that NAL infusion increased NPY levels and release, while it concurrently promoted LHRH levels and release[31] as well as adrenergic turnover and release,[24] imply that the EOP-NPY connection is important in the induction of preovulatory discharge of LH. Consequently, we proposed that the NC gradually decreases the tonic inhibitory opioid tone in the hypothalamus sometime before the critical period on proestrus. In response to this decrease in opioid influence, there is increased activation of NPYergic and adrenergic systems which, in turn, on a temporal basis, stimulate the episodic discharge of LHRH. A sustained stimulation of the pituitary by LHRH in cooperation with NPY results in a massive discharge of LH in the afternoon of proestrus.

NPY and Aging

Gonadotropin and testosterone (T) secretion is markedly suppressed during reproductive aging in male rats.[32,33] We observed that the KCl-induced NPY release *in vitro* from the MBH of 11-month-old rats was decreased and there was a widespread suppression of NPY levels in 7 hypothalamic nuclei, including the ARC, ME, and MPOA in 15-month-old rats.[32] Since NPYergic systems are excitatory to LHRH, these studies support the view that the age-dependent decrease in the NPY excitatory input in the hypothalamus leads to depressed secretion of LHRH and LH which, in turn, are responsible for reduced gonadal function.[32]

Regulation of NPY Secretion by Gonadal Steroids

Several lines of evidence suggest that gonadal steroids modulate NPY secretion in male and female rats. We observed that two weeks after castration, hypothalamic NPY release *in vitro* in response to K^+ decreased and of the 6 microdissected hypothalamic sites, NPY levels were reduced selectively in the ME, ARC, and VMH.[34] Testosterone (T) replacement in the physiological range to castrated rats restored the NPY release response as well as levels in these three sites.[35] It appears that steroid-concentrating cells

in the BS and hypothalamus may participate in these site-specific stimulatory actions of T.[36] When the BS projections to the hypothalamus were interrupted, T replacement was still effective in raising NPY levels in the ARC and VMH, but not in the ME. Apparently, the site of T action is different in modulating NPY levels in these three hypothalamic sites. To augment NPY levels in the ME, T may require an intact BS-hypothalmic link, whereas in the VMH and ARC, the site of T action may be resident locally in the hypothalamus.[36]

Our more recent studies show a sexually dimorphic response in the ME NPY levels and release.[37] Unlike in male rats, ovariectomy failed to change either the basal or K^+-induced MBH NPY release *in vitro* and decreased NPY concentration in the ARC and VMH, but not in the ME. E_2 replacement restored NPY levels in the VMH and ARC, but in the ME the levels were slightly decreased.[37] Cumulatively, it is clear that gonadal steroids modulate NPY secretion and despite a common source of hypothalamic NPY in male and female rats, there is a sexually dimorphic NPY response in the ME. Unlike in male rats, NPY levels do not decrease in the ME of female rats in response to gonadectomy and ovarian steroids evoke marked fluctuations in association with the LH surge in ovx rats.

Where is the cellular site of gonadal steroid action? We suspect that a population of neurons that possess steroids receptors, namely in the MPOA, VMH and ARC and those in the BS[38,39] are likely to modulate NPY neurosecretion. In collaboration with Dr. M. Sar (University of North Carolina), we have recently observed co-localization of ^{3}H-estradiol-17β and NPY immunoreactivity in a certain population of cells in the ARC. The proportion of cells showing co-localization of E_2 and NPY varied (5–25%) from the anterior to posterior segment of the ARC. Consequently, direct genomic activation in NPY neurons in the ARC may, in part, be responsible for facilitation of NPY neurosecretion by gonadal steroids in male and female rats.

NEUROPEPTIDE Y AND FEEDING BEHAVIOR

During the course of our studies to investigate the effects of human pancreatic polypeptides and NPY on pituitary LH release in 1982–83,[1,2,40,41] robust feeding activity was observed in rats after the intraventricular administration of these peptides.[42,43] We have extended these unexpected findings, affirmed by Stanley *et al.*[44] and Levine and Morley,[45] to test the hypothesis that NPY may be one of the key physiological signals that encodes acquisition of daily food intake in rats.

Effects of NPY on Feeding Behavior

In general, extremely low NPY concentrations that are well within the physiological range, evoke a dose-related feeding response in satiated male and female rats.[42,43,46,47] NPY is equally effective during the daytime when normally rats do not eat, and during the nighttime when they feed voraciously. The feeding induced by NPY is generally of a discontinuous nature, and the dose-related increase in feeding is largely due to an increase in the duration of feeding and the local eating rate (g/min). Continuous intraventricular infusion of NPY in satiated female rats also stimulated a dose-related feeding response during the 4-h infusion period, and these rats continued to eat albeit at reduced levels, during the 2-h postinfusion period.[48–50] Seemingly, an extremely low level of NPY receptor activation can sustain the motivation to acquire food for a considerable period of time in satiated rats, and the rapid fall in the rate of food consumption after cessation of NPY infusion, can be attributed to a decrease in NPY receptor activation rather than

satiety. Also, we observed that continuous NPY infusion modified the microstructure of feeding behavior. Feeding occurred in discrete episodes, and time spent eating was increased by NPY infusion. Since the episodic stimulation of feeding by NPY bears a strong resemblance to the normal intermittent nocturnal feeding pattern in the rat,[51,52] it is reasonable to suggest that "an increase in NPY secretion may normally be responsible for the sustained intermittent nocturnal feeding pattern, and that the onset of the dark phase may, itself, herald activation of NPY release in relevant brain sites."[48-50] The following studies concur with this hypothesis.

Effects of Food Deprivation (FD) on Hypothalamic NPY Output

If NPY is a neurochemical signal that stimulates "hunger" or "appetite" for food, then one would expect an increase in NPY activity in relevant target brain sites in response to FD. Indeed, our investigation of the time course of changes in NPY levels in the PVN in response to FD followed by food *ad libitum*, attests to this view.[53] We observed that of the 7 hypothalamic sites, the PVN was the only site where NPY levels fluctuated in a predictable reciprocal manner in response to FD and food intake. FD produced a steady accumulation of NPY in the PVN reaching a plateau by day 3 and thereafter, in response to *ad libitum* supply of rat chow for one day, which apparently induced a satiety state, the PVN NPY concentrations returned to the control range. In addition, in the ARC where NPY perikarya are localized, NPY levels increased steadily during FD and remained elevated even after one day of *ad libitum* food supply. That the PVN NPY response is specifically related to ingestive behavior is indicated by the finding that NPY levels were unaffected by FD in the ME, the other terminal bed of NPY perikarya located in the ARC.[53]

Although the estimation of NPY levels in the PVN reflects a balance of release, synthesis and metabolism of the neuropeptide, these observations are in accord with the proposal that FD may increase the supply of NPY to the PVN, as a result of an enhanced rate of NPY synthesis. The observation of increased Pre-Pro NPY mRNA in response to FD[54] are in line with this proposal and further reveals that the experimentally-induced "appetite" selectively activates the ARC-PVN NPY line of communication. In addition, we observed that the PVN was the only site where NPY levels returned to the normal range after presentation of *ad libitum* food. This selective specific response suggested that NPY receptive elements concerned primarily with elicitation of ingestive behavior are located in the PVN.[53,55]

Effects of Experimental Diabetes on NPY Output

If one assumes that NPY, the most potent orexigenic peptide known, is an endogenous signal to normally evoke nocturnal feeding, then it is reasonable to suspect that abnormal NPY neurosecretion may be the underlying cause of eating disorders due to disease and stress, and the hyperphagia and obesity produced by genetic factors. Recent evidence shows that NPY concentrations in many hypothalamic sites increase after experimentally-induced diabetes.[56,57] NPY concentrations increased in the PVN and other neighboring sites for up to 6 months after streptozotocin injection (STZ) and these effects were reversed with insulin substitution therapy.[57] In addition, the *in vitro* release of NPY in response to K^+ depolarization from the hypothalami of STZ-diabetic rats was significantly increased.[57] In these rats, White *et al.*[58] found evidence of increased NPY gene expression in the ARC. Additional studies suggested that insulin *per se* may modulate NPY neurosecretion in the ARC-PVN axis.[57] Presumably then, irregularities in insulin

secretion may influence NPY output in the PVN and the increased NPY secretion may be the underlying cause of hyperphagia in STZ-treated diabetic rats.

Effects of Experimentally Altered Hypothalamic NPY Secretion on Feeding Behavior

An altered pattern of eating behavior or reduced appetite is a common symptom of central nervous system diseases, drugs of abuse and neuroactive agents. We speculated that if NPY is an endogenous orexigenic agent then altered NPY secretion may be the underlying cause of changes in eating behavior patterns under these conditions. To meet this objective, we assessed the effects of selectively decreasing the BS NPY input into the hypothalamus on eating behavior in a series of experiments in the rat. Adult male rats underwent bilateral neural transection at the level of the dorsal tegmental area of the mesencephalon.[14] This surgical transection drastically reduced hypothalamic adrenergic transmitters and reduced NPY levels of BS origin by more than 50% selectively in the PVN, MPOA, ME and dorsal medial nucleus. As a consequence of this decreased NPY and adrenergic input, rats were rendered hyperresponsive to NPY.[59] In general, the latency to onset of feeding was significantly reduced, these rats ate sooner than controls in response to NPY. Food consumption was augmented to the extent that low doses of NPY evoked maximal food intake thereby the dose-related consumption seen in normal rats was abolished in transected rats. We propose that this hyperresponsiveness of two parameters of feeding in these neurally transected rats reflects denervation-induced supersensitivity caused by reduced NPY release in the PVN. Furthermore, it is likely that this hyperresponsiveness to NPY is specifically mediated by NPY receptors because despite concurrent reduction in adrenergic transmitters, a similar hyperresponsiveness in feeding parameters was not evident after NE injection. These observations are consistent with the possibility that altered function of brain NPY may mediate the pathophysiology of eating disorders associated with stress, central nervous system diseases and drugs of abuse.

Structure-Function Relationship

Several members of the pancreatic polypeptide family—human pancreatic peptide, rat pancreatic peptide and PYY—are effective in stimulating feeding in the rat. Each of these members of the PP family of peptides elicited a reliable dose-related increase in food intake, but the cumulative response and the parameters of eating were differently altered.[42,43,46] Nevertheless, their effectiveness after central administration raise the possibility that these pancreatic polypeptides may bind to NPY receptors in the brain to evoke feeding and that each of these peptides may contain a sequence of amino acids readily recognized by NPY receptors in the rat brain. In our quest to identify the sequence of the amino acid in the parent NPY molecule that stimulates feeding, we observed that the first amino acid at the N-terminal of NPY is not essential for stimulation of feeding (unpublished). In fact, NPY_{2-36} was more effective than the parent NPY_{1-36} in stimulating cumulative food intake. Additional deletions of amino acids at the N-terminal end rendered the peptide fragment completely ineffective in stimulating feeding behavior. Further [Leu^{31},Pro^{34}] NPY, a specific Y_1 receptor agonist[16] and not NPY_{13-36}, a Y_2-receptor agonist[17] stimulated food intake in satiated male rats. Presumably, increased food intake in response to endogenous NPY and, possibly, other members of the pancreatic polypeptide family, is mediated postsynaptically by Y_1 NPY receptors.

CONCLUDING REMARKS

We have simultaneously studied the involvement of NPY messenger molecule in the control of a neuroendocrine and a behavioral response in the rat. Our findings show that NPY is a key component of the hypothalamic neural circuitry that controls reproduction and appetitive behavior. Despite the common origin of NPY, it acts at specific and discrete hypothalamic sites to modulate these two diverse responses. A primary source of NPY for these responses is the population of NPY producing cells in the ARC of the hypothalamus. Projections from these NPY cells to the preoptic-tuberal pathway regulate the LHRH-LH axis, and those to the PVN stimulate ingestive behavior. Apparently, both the neuroendocrine and behavioral responses are evoked by activation of postsynaptic Y_1 NPY receptor subtypes. We propose that additional regulatory systems may play a key role in regulation of NPY output locally in the target sites and that disease states, *e.g.*, diabetes, and environmental factors, *e.g.*, stress, that affect biosynthesis, processing and transport of this messenger molecule from cell bodies in the ARC may cause multiple neuroendocrine and behavioral abnormalities.

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Hypothalamic Neuropeptide Y in Relation to Energy Balance[a]

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A novel peptide, neuropeptide Y (NPY), with a C-terminal tyrosine amide, was isolated from the brain in 1982.[1] This peptide, named neuropeptide Y (NPY), has 36 amino acids, contains five tyrosine residues in its primary structure, and has structural similarities to peptide YY (PYY) and pancreatic polypeptide. Following the isolation of NPY, a variety of studies has demonstrated that NPY is widely distributed throughout the central and peripheral nervous systems and has a multiplicity of physiological functions, including the regulation of neuroendocrine secretion and circadian rhythmicity, modulation of consummatory and reproductive behavior, and sympathetic cardiovascular control. The structure of NPY has been well conserved during evolution, and studies of the structure-function of NPY indicate that the C-terminal amide structure of NPY is critical for its biological activity. Moreover, NPY is found to act at both the pre- and postjunctional levels, and the whole structure of NPY appears to be required for its postjunctional effects, while a C-terminal portion of the molecule is sufficient for exerting the prejunctional effects.

This review will concentrate on the effects of NPY on consummatory behavior and associated endocrine and metabolic systems. Neuropeptide Y is known to have particularly high concentrations within the hypothalamus, a structure which is most critically involved in energy homeostasis and neuroendocrine/autonomic systems. This peptide is also known to coexist, in certain hypothalamic neurons, with the classical aminergic neurotransmitters, namely, norepinephrine (NE), epinephrine (EPI) and serotonin (5-HT). Recent studies, building on extensive evidence showing NE and 5-HT to have potent and well-defined effects within the hypothalamus,[2,3] suggest that these hypothalamic neurocircuits may provide excellent model systems for examining, in localized brain areas, the precise nature of the peptide-amine interactions in the brain and their physiological function in controlling ingestive behavior and specific endocrine and metabolic processes.

A wide variety of evidence now indicates that most neurons utilize more than one transmitter to perform their functions. This neuronal coexistence of transmitters greatly increases the number of messages that reach the postsynaptic cell, to affect its metabolic and electrical state. Through immunohistochemical techniques, certain peptides have been identified and found to coexist with the monoamines in neurons and neurosecretory cells of the periphery and brain.[4] These neuropeptides and the classical neurotransmitters may be colocalized in the same or different subcellular organelles within the nerve terminal, and investigators have attempted to determine the nature of the interaction between the neurotransmitters and their receptors. The peptides may act directly via their own peptidergic receptors and independently of the monoamines; they may also act in a dependent fashion, either presynaptically through modulating the release of the monoamines or postsynaptically through modulation of the monoaminergic effector response or

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through a direct receptor-receptor interaction. There is evidence for each of these possibilities, and the specific role, relative importance, and mode of interaction of the different coexisting messengers in the overall effector response appears to vary with the geometry of innervation and the receptor characteristics of the specific tissue under investigation.

The effects of NE and 5-HT in the hypothalamus have been extensively studied, and the evidence obtained has established specific functions for these endogenous monoamines in the physiological control of energy balance and neuroendocrine processes. To provide an appropriate background for understanding the actions of NPY, an overview of this evidence, in which the monoamine effects are related to natural patterns of eating behavior, will be presented first. The findings obtained with NPY will then be described and related to the actions of the monoamines, in an attempt to define the nature of their interaction in the physiological control of energy balance.

Characterization of Natural Patterns of Eating Behavior

To define the precise physiological function of NPY, as well as the amines, it is essential that the natural patterns of feeding and related responses first be characterized as fully as possible, both temporally and qualitatively in terms of the specific nutrients being consumed. Since the feeding response under one condition is likely to be different from the response under another condition, the underlying neurotransmitter substrates are also likely to differ. With precise definition of the relevant conditions and of the nature of the response expressed under these conditions, there is some hope that the physiological function of the various transmitters and hormones in controlling normal behavior can be identified. With this information available to us, our ultimate goal, that of determining the disturbances in brain neurotransmitters associated with abnormal eating patterns in humans, becomes more obtainable.

Most species exhibit a strong circadian pattern of feeding behavior. The rat, a nocturnal animal, consumes most of its food in the dark cycle, exhibiting periods of intense feeding activity particularly at the beginning and the end of the dark. The feeding that occurs during these early and late dark periods differs in several respects, suggesting that the responses at these two times may be differently regulated.[5-10] For example, rats maintained on pure macronutrient diets display a strong preference for carbohydrate at the onset of the dark period, in contrast to a preference for protein toward the end of the dark. Moreover, they exhibit temporal differences in their responsiveness to experimental conditions, with stronger compensatory feeding to deprivation occurring at the beginning of the feeding cycle and a decline in the satiating value of food apparent towards the end of the feeding cycle.

These temporal differences in feeding behavior may reflect a dependency of early dark feeding on immediate energy requirements, as opposed to an anticipatory quality of late dark feeding which must provide energy resources for later utilization during the light period. Since carbohydrate has the most direct and rapid effects on blood glucose,[11] the rats' preference for this nutrient during their first meal of the active cycle may serve to restore body energy levels reduced during the inactive period. Moreover, the stronger satiating value of protein[9] may explain the rats' preference for this nutrient toward the end of the active cycle.

More detailed analyses of meal patterns, using computer-assisted techniques, have revealed in freely feeding rats a gradual decline in consumption of carbohydrate across the 12 hours of the nocturnal cycle, in association with a gradual rise in preference for protein and to a lesser extent fat.[8-10] The first meal in most rats is carbohydrate predominant, occurring within 10 minutes after the lights go out; this contrasts with meals later in the

nocturnal cycle, when preference for carbohydrate decreases and protein or fat intake predominates.

Superimposed on this gradual shift across the 12-hour nocturnal cycle, there also appears to be a natural, meal-to-meal shift in nutrient preference.[9] The first carbohydrate-rich meal, which lasts 10–15 min, is followed by an intermeal interval of 30–60 min and then a second meal that shows an increase in protein content along with a decrease in relative concentration of carbohydrate. This meal-to-meal shift at dark onset is apparent throughout the entire night, with a meal predominant in a specific nutrient preceded and followed by a meal that is low in that nutrient. This natural meal pattern suggests that different neurochemical or humoral mechanisms exist to mediate the different meals consumed over the course of the active cycle and possibly to induce nutrient-specific satiety.

Norepinephrine

Effects on Eating Behavior

Hypothalamic stimulation with NE or EPI has long been known to elicit feeding in satiated animals.[12,13] Studies in brain-cannulated rats demonstrate that this phenomenon has a rapid onset (<1 min) and a short duration (<15 min); it is anatomically localized, to the hypothalamic paraventricular nucleus (PVN), in addition to the periventricular region and ventromedial hypothalamus (VMH), and it is mediated via α_2-, rather than α_1-, type receptors located on the postsynaptic membrane.[14–16] Destruction of the PVN, as opposed to other brain sites, disturbs the animal's ability to respond to NE,[13,17] as well as to regulate normal circadian patterns of carbohydrate and protein intake.[18] Moreover, biochemical studies, employing the push-pull, microdialysis and micropunch techniques to measure endogenous NE in the PVN or VMH,[19–25] have revealed an enhanced release or turnover of NE in association with a spontaneous or deprivation-induced feeding response. The opposite pattern, a decline in NE efflux, has been observed after the meal occurs or in response to an intragastric nutrient load.

The primary effect of NE, or the selective α_2-receptor agonist clonidine, is to cause a preferential increase in the animal's appetite for carbohydrate, generally at the expense of protein intake, and to enhance the size of the carbohydrate meal ingested, rather than the frequency of meals taken.[17,26–28] A chronic state of overeating and weight gain, with a strong preference for carbohydrate and a reduction in protein intake, can be induced by continuous or phasic infusion of NE or clonidine directly into the PVN or VMH.[26,28–32] In contrast, a deficit in food intake, particularly carbohydrate ingestion, and a decrease in body weight occurs in response to local or peripheral infusion of agents which block α_2 receptors, inhibit NE synthesis or destroy noradrenergic innervation.[17,32–36]

Diurnal Rhythm

The activity of this α_2-noradrenergic system in the PVN is closely linked to the light/dark cycle. The available evidence suggests that NE in the PVN may be most active in stimulating eating, in particular carbohydrate intake, specifically at the *onset* of the active feeding period. At this time, the feeding responses induced by NE or clonidine are found to be considerably larger than at other times later in the dark cycle or during the light period.[37–39] Moreover, a microdialysis study of endogenous NE in the PVN has revealed a single, circadian peak of extracellular NE levels at dark onset[40] (FIG. 1), while receptor binding studies have shown a dramatic rise in medial hypothalamic or PVN α_2

receptor sites at dark onset.[41,42] This increase in α_2-noradrenergic activity coincides with the strong carbohydrate preference exhibited naturally by rats in the early dark period.[8-10] It is proposed that endogenous NE has an active role in mediating the first carbohydrate-predominant meal which occurs within 10 min after dark onset.[17]

Hormones and Metabolism

Evidence is now accumulating to suggest that the activity of PVN NE in stimulating carbohydrate ingestion is closely associated with circulating hormones, in particular corticosterone (CORT), insulin and vasopressin, all of which are known to control the level

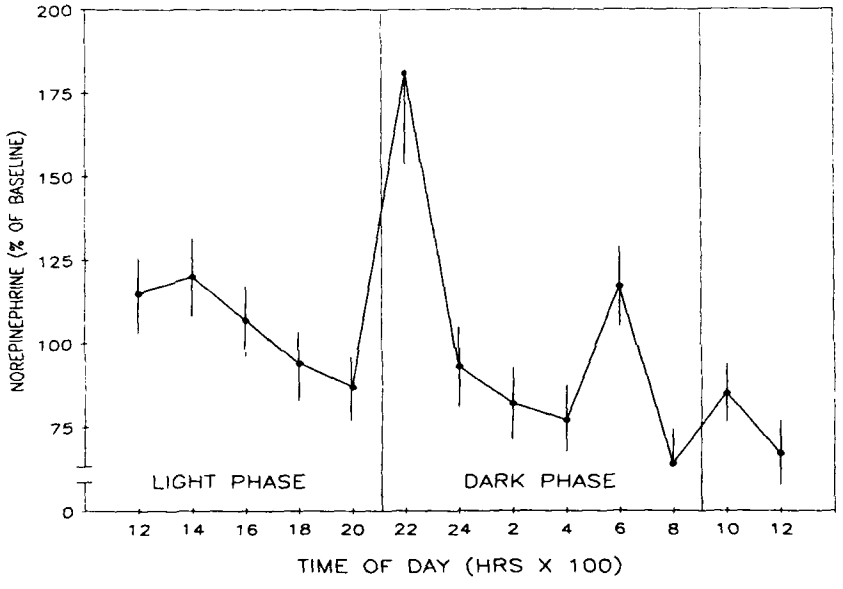

FIGURE 1. Pattern of Extracellular norepinephrine (mean ± SEM) in 11 freely feeding rats as a function of time of day. The NE levels at 22:00 hrs are significantly greater ($p < 0.05$) than at any other time. (From Stanley *et al.*[24] Reprinted by permission from *Life Sciences*.)

and metabolism of circulating and stored carbohydrates.[12,13,17,43] Medial hypothalamic or PVN injection of NE or EPI increases circulating levels of each of these hormones, in addition to blood levels of glucose.[44,45] Moreover, these hormones and nutrient are additionally found to feedback positively on the PVN α_2 receptors, to enhance the action of NE in stimulating feeding and in preferentially increasing appetite for carbohydrate.[17] Similar to the temporal pattern described above for natural and NE-induced carbohydrate feeding, the endogenous release pattern and feedback action of these hormones is closely linked to the light/dark cycle, peaking at the onset of active cycle. This diurnal rhythm of neuroendocrine function may, in part, be attributed to the unimodal peak of endogenous NE and α_2-receptor activity observed in the PVN at onset of the feeding cycle.[10,17,40,41,46]

In addition to this association between PVN NE and circulating hormones in coordinating natural feeding patterns, there is evidence that NE may also have direct modulatory effects on metabolism. Norepinephrine in the PVN is found to cause a reduction in energy expenditure in freely moving as well as lightly sedated rats.[47] This effect occurs rapidly and in the absence of any change in locomotor activity. Together with the above findings revealing a stimulatory effect of NE on energy intake and the release of glucoregulatory hormones, this evidence on energy expenditure supports a role for PVN and possibly VMH noradrenergic neurons in coordinating various processes geared towards energy conservation, most particularly at the beginning of the active feeding period when energy stores are most severely depleted.

Physiological Function of Medial Hypothalamic NE

As described above, NE injected into the PVN has a variety of behavioral, endocrine and physiological effects relating to energy balance. Moreover, the endogenous NE and α_2-noradrenergic receptors that mediate this effect are themselves modulated by metabolic and endocrine substances which fluctuate depending upon the physiological state of the animal and upon the diurnal rhythm. Taken together, the evidence suggests that these phenomena revealed experimentally reflect the normal functioning of this α_2-noradrenergic system in the PVN.

The PVN, through its anatomical and neuroendocrine processes,[48] is known to receive and integrate multiple inputs from metabolic, hormonal and neural factors regarding the nutritional status of the animal, and then translate this information into appropriate signals for maintaining internal homeostasis. The knowledge we have obtained, from studies of natural daily rhythms, of short-term changes induced by deprivation, hormones and nutrients, and of the effects caused by central injections of NE, has led us to formulate the following hypothesis regarding α_2-noradrenergic function in the PVN.

It is proposed that NE, through the activation of glucocorticoid- and glucose-sensitive α_2-receptor sites in the PVN (and possibly the VMH), is physiologically active in energy homeostasis, particularly at the onset of the animal's active cycle and specifically during the first meal of this period. This neurotransmitter in the PVN, derived from projections ascending from the locus coeruleus and medullary cell bodies, appears to evoke a state of energy conservation.[17] This state involves adjustments in carbohydrate ingestion as well as metabolism that allow animals to maintain energy reserves by anticipating or responding to a depletion. These adjustments in endogenous NE turnover and α_2-receptor activity may occur, in conjunction with fluctuations in CORT, glucose and vasopressin, to determine the precise nature and time course of meals consumed.

Serotonin

Effects on Eating Behavior

In contrast to NE or EPI, hypothalamic administration of serotonin (5-HT) has a suppressive effect on food intake and body weight in freely feeding or food-deprived animals.[49-53] This effect occurs with both peripheral and central administration of serotonergic drugs which are believed to act through the release of endogenous 5-HT. Cannula mapping and lesion studies indicate that this phenomenon is anatomically localized to the medial hypothalamus, specifically the PVN and VMH, in addition to the suprachiasmatic nucleus (SCN). Pharmacological tests suggest that it is mediated by postsynaptic 5-HT_{1B}

receptor sites, which are found to be particularly dense in the medial hypothalamic nuclei[54] where endogenous 5-HT is most active in controlling food intake.

The serotonergic system, similar to the α_2-noradrenergic system, has a selective effect on macronutrient intake.[7,49-52] Serotonin in the PVN, as well as the VMH and SCN, dose-dependently suppresses carbohydrate intake, while sometimes enhancing appetite for protein, and it reduces the size rather than the frequency of the carbohydrate meals consumed. This evidence is consistent with other findings obtained with lesions in these three nuclei, demonstrating the essential role of these hypothalamic sites in controlling satiety for carbohydrate, the ratio of carbohydrate/protein in the diet, as well as the animals' responsiveness to peripherally-injected serotonergic drugs.[18,49,50,55] It also agrees with the results of studies with the serotonergic agent, d-norfenfluramine, which acts through the release of endogenous 5-HT and mimics the feeding-inhibitory action of exogenous 5-HT in these medial hypothalamic areas.[50,51,56,57] As revealed via the microdialysis technique, microinjection of d-norfenfluramine directly into the PVN causes a significant enhancement of extracellular levels of endogenous 5-HT.[57]

Diurnal Rhythm

As indicated for NE, the activity of the PVN serotonergic system shifts across the light/dark cycle, exhibiting a peak at the onset of the active feeding period. This rhythm is reflected in pharmacological experiments, showing hypothalamic injections of 5-HT or the serotonergic drugs to be most effective and selective in suppressing carbohydrate intake at the start of the natural feeding cycle.[49,52] It has also been demonstrated in biochemical studies of 5-HT synaptic content, synthesis, metabolism or uptake in the PVN, SCN or VMH, as well as in radioligand binding assays of hypothalamic 5-HT_{1B} receptors, which are found to peak in density either a few hours prior to (PVN and SCN) or precisely at (VMH) the onset of the dark cycle.[58-64]

As described above, carbohydrate-rich meals consumed under natural feeding conditions predominate during the early hours of the feeding cycle.[8-10] Thus, it is proposed that 5-HT in the medial hypothalamus plays a specific role in terminating these initial carbohydrate meals, possibly by stimulating PVN or VMH "satiety" neurons known to control intake of this macronutrient,[18,55] as well as SCN neurons that determine the circadian rhythms of physiological systems.[58,60-64]

Physiological Function of Medial Hypothalamic 5-HT in Relation to NE

Based on the above evidence, showing NE and 5-HT to have opposite effects on food intake, nutrient appetite, meal patterns, and consequently body weight gain, it has been proposed[13,49] that 5-HT in the medial hypothalamus interacts antagonistically with NE to modulate the circadian pattern of feeding and, in particular, to control the sequence of carbohydrate and protein ingestion specifically at the beginning of the animal's active cycle. The neural substrates for this 5-HT modulation originate from the midbrain dorsal raphe nuclei and include: 1) serotonergic projections to the PVN and VMH, where there exist neurons that control satiety in relation to the body's energy stores, and 2) serotonergic projections to the SCN, a primary circadian pacemaker that controls temporal patterns of feeding, possibly independently of energy requirements.

The possibility that 5-HT and NE interact directly, on the same medial hypothalamic neurons controlling satiety for carbohydrate, is supported by studies demonstrating a reversal of the NE-elicited eating response with PVN injection of 5-HT at low doses or of drugs which release endogenous 5-HT.[49,53] Further evidence indicates that the 5-HT

precursor, 5-hydroxytryptophan, operating through the synthesis of endogenous 5-HT, is similarly effective in antagonizing NE's action in the PVN.[49] This interaction between serotonergic and α_2-noradrenergic receptor stimulation in the PVN is consistent with the results of electrophysiological studies, which have shown 5-HT to have a predominantly excitatory effect on PVN neuronal activity in contrast to the inhibitory action of NE,[65] and also the results of biochemical studies, that have revealed a direct antagonism between these neurochemical systems in the rat hypothalamus.[66]

Thus, rather than tonically inhibiting feeding behavior, endogenous 5-HT is believed to act phasically, according to a distinct daily rhythm with a peak at the beginning of the active feeding cycle.[50,67] At this time, rats under freely-feeding conditions are found to consume a large, initial meal that is rich in carbohydrate and frequently absent of protein. This meal is then followed by a second, smaller meal that is rich in protein. It has been proposed[17] that the first carbohydrate meal is stimulated, in part, by activation of the α_2-noradrenergic system in the PVN. While relatively inactive at the beginning of this meal, 5-HT in the medial hypothalamus may increase its activity gradually over the course of this carbohydrate meal, due to enhanced tryptophan uptake into the brain, and then, through the stimulation of 5-HT_{1B} receptors and antagonism of α_2-noradrenergic activity, 5-HT evokes a state of satiety that is specific to carbohydrate. In view of its tendency to have opposite effects on protein intake,[67] 5-HT may additionally play a role in switching the animal's preference towards protein, thereby balancing the ratio of the two macronutrients in the diet. Protein is naturally preferred in the second meal of the feeding cycle, and its ingestion has sometimes been shown to be enhanced by 5-HT while reduced by NE or clonidine.

Neuropeptide Y

Effects on Eating Behavior

In addition to containing NPY receptor binding sites,[68] the hypothalamus, and in particular the PVN, is known to receive a rich innervation of NPY-immunoreactive nerve endings, either from EPI-containing neurons in the medulla (C1 and C2), NE-containing neurons in the medulla (A1) and dorsal pons (A6), or noncatecholaminergic neurons in the arcuate nucleus.[69] When injected directly into the PVN, a primary site of action for NE and EPI,[15] the eating response induced by NPY, as well as by the related substance peptide YY (PYY), is found to be dose dependent, within the range of 0.02 to 1.0 nmole.[70] The maximal feeding response induced by an optimal dose of NPY or PYY (0.25 nmol) in satiated rats is 15 g in 1 hour, which is approximately 50% of the rat's normal daily intake (FIG. 2).

With measurements taken during the first hour after injection, the maximal feeding responses elicited by NPY and by NE or EPI in the PVN are comparable in magnitude.[44,71] However, the latency of these two responses differs considerably, with the amines acting within <1 min[15] and NPY after 10 min.[72] Moreover, their response duration also differs, <30 min for NE and >4 hrs for NPY, which allows NPY to yield a more potent, long-term effect.[72] This difference in duration of action has similarly been detected in studies of the inhibitory effect of PVN NE and NPY on gastric acid secretion,[73] as well as in studies of peripheral autonomic function.[74,75]

Similarities between the Effects of NPY and NE

The behavioral and endocrine responses elicited by NPY and NE in the PVN are similar in a number of respects. In addition to producing a small increase in drinking

behavior, both substances stimulate food intake by potentiating the rate of eating, as well as the duration of the eating response.[76,78] They also increase preferentially the rat's intake of carbohydrate, while having little or no effect on protein or fat ingestion[26,70,79] (FIG. 3).

Moreover, both substances appear to be most active at the onset of the nocturnal (active) cycle. They produce their largest feeding response at this time, as compared to a relatively weak response at the end of the nocturnal feeding period.[2,38] As with NE, this peak behavioral responsiveness to PVN injection of NPY occurs simultaneously with a unimodal rise in endogenous NPY content or synthesis in the PVN.

The NPY-elicited feeding response also appears to be closely linked to circulating levels of the adrenal hormone, CORT, which rise towards the onset of activity. Similar to NE and EPI, NPY in the PVN stimulates the release of CORT,[44,80] as well as of vasopressin[44] (FIG. 4). In addition, the feeding response elicited by NPY, as with NE,[17] is dependent upon circulating CORT levels which naturally peak at dark onset.[81]

This evidence, revealing considerable similarities between the behavioral and endocrine effects of NPY and NE (or EPI) in the medial hypothalamus, suggests that this peptide and amine function, at least to some extent, via common neural mechanisms to control energy balance, in particular, carbohydrate feeding and possibly metabolism.[2] However, a variety of pharmacological, anatomical and biochemical evidence, described below, indicates that NPY can act largely independently of NE and that its action may actually be potentiated in response to depleted stores of endogenous NE.

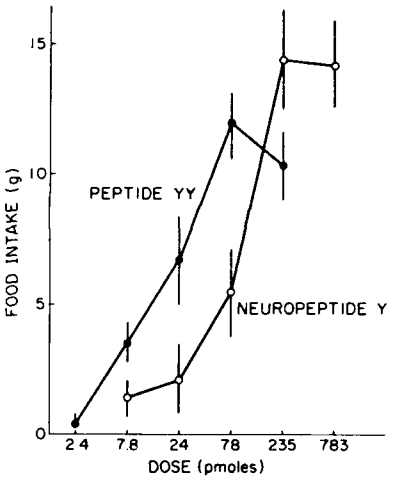

FIGURE 2. Food intake (mean ± SEM) 60 min postinjection, by rats given PVN injections of PYY (*closed circles*, n = 7) or NPY (*open circles*, n = 10). (From Stanley *et al.*[70] Reprinted by permission from *Peptides*.)

Relationship and Interaction between Neuropeptide Y and Norepinephrine

A number of studies indicate that the actions of NPY and NE in the hypothalamus can be dissociated.

Pharmacological Studies

Pharmacological evidence, obtained in a variety of peripheral and central systems, has generally indicated that NPY, while mimicking the action of NE or EPI, acts via its own

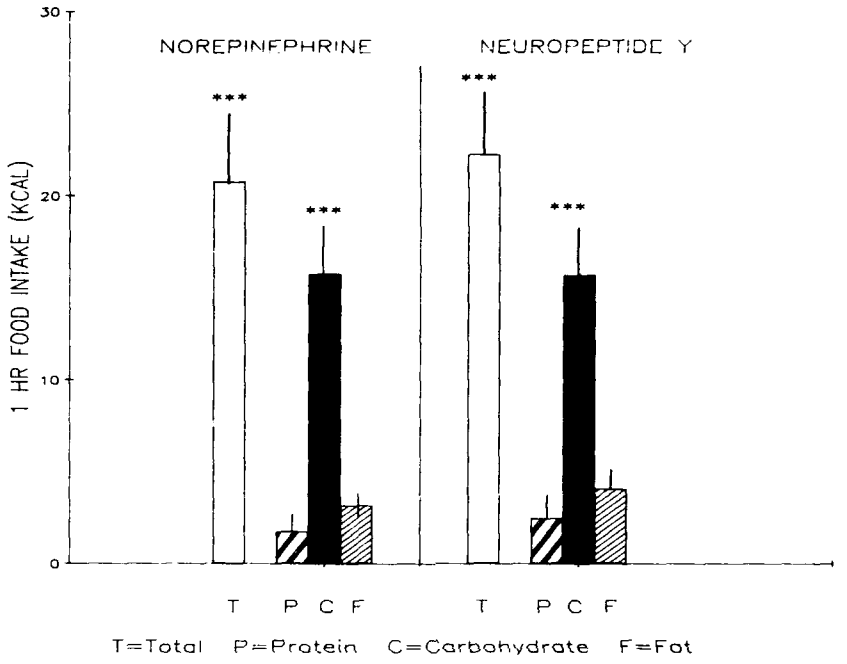

FIGURE 3. Macronutrient intake (Kcal) 60 min postinjection in rats given PVN injection of NE (40 nmol) or NPY (78 pmol). ***$p < 0.001$ relative to baseline.

peptide receptor and independently of the postsynaptic α-noradrenergic receptor site.[74,75,82,83] This also appears to be the case with the feeding-stimulatory effect of NPY in the PVN. This eating response is unaffected by local administration of general α-noradrenergic or selective α_2-noradrenergic receptor antagonists,[72,84-86] which abolish eating elicited by NE[14] (FIG. 5). It may, in fact, be potentiated by peripheral administration of an α_2-receptor antagonist,[84] as well as by PVN injection of the monoamine 5-HT (Kyrkouli *et al.*, unpublished data) which is found to inhibit the feeding response elicited by NE.[53]

In addition to their independent action on the postsynaptic membrane, other evidence indicates that NPY elicits feeding independently of presynaptic stores of NE, consistent with findings obtained in other systems.[4,74,75,82] Specifically, the feeding stimulatory effect of NPY remains intact, and may in fact be significantly potentiated, after local PVN administration of catecholamine synthesis inhibitors,[2,85,87] as well as after brainstem knife cuts which reduce hypothalamic NE and EPI levels.[84] These manipulations, which damage adrenergic afferents to the PVN, are both effective in antagonizing the behavioral effects of catecholamine-releasing drugs in this nucleus.[12,16,88] Thus, NPY, in modulating food intake, does not appear to require the functional release of NE from presynaptic nerve endings in the PVN.

Neuroanatomical Studies

Additional evidence dissociating the action of NPY and NE derives from mapping experiments that tested the sensitivity of multiple brain sites to NPY injection. These

studies have demonstrated that, while NE acts in a localized fashion within the area of the PVN to stimulate food intake,[15] the eating response produced by NPY administration can be observed throughout multiple hypothalamic sites, although generally not in extrahypothalamic areas.[79,89]

However, the most recent evidence suggests that the PVN, in particular its caudolateral portion, is at least one of this peptide's main sites of action.[90] In this extensive cannula mapping study, NPY was injected into the hypothalamus in a particularly small volume of 10 nl. With this method, the hypothalamic sites most responsive to NPY injection were found to be located at the level of the PVN, and by far the largest response was obtained in the caudolateral part of the PVN, as well as in the medial perifornical region just lateral to the PVN. At this site, the animals consumed over 12 grams of food in 1 hour and 20 grams in 4 hours, which is >60% of the rats' normal 24-hour intake. Injections of NPY into sites bracketing this region, less than 1 mm from the critical area, yielded significantly reduced responsiveness. This degree of anatomical definition is consistent with additional analyses showing the distribution of ^{125}I-NPY, after hypothalamic injection, to be essentially restricted to a 0.5-mm radius. This confirms the importance of the caudolateral part of the PVN in the mediation of NPY's action. This is in

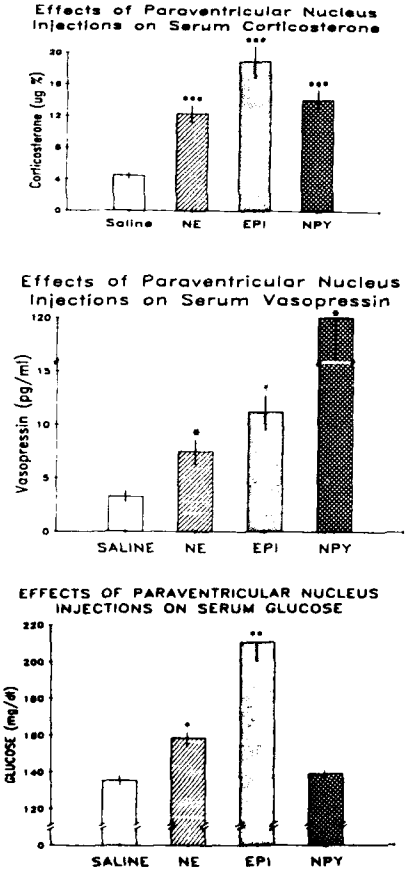

FIGURE 4. Serum levels of corticosterone (μg%), vasopressin (pg/ml), and glucose (mg/dl) measured 15 minutes after PVN infusion of saline vehicle, norepinephrine (NE), epinephrine (EPI) or neuropeptide Y (NPY). ***p <0.001 relative to saline baseline. (From Leibowitz *et al.*[44] Reprinted by permission from *Brain Research Bulletin.*)

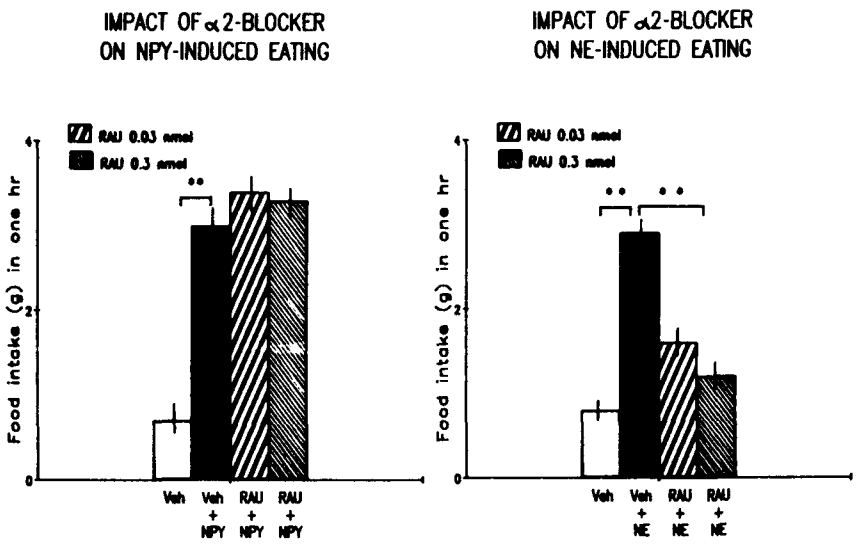

FIGURE 5. Impact of PVN injections of the α_2-receptor blocker rauwolscine (RAU) on the feeding response induced by local administration of neuropeptide Y (NPY <78 pmol) or norepinephrine (NE, 63 nmol) in satiated rats. $^{**}p < 0.01$.

distinct contrast to the medial, periventricular portion of this same nucleus which is believed to be most critical in the mediation of the eating response associated with NE.[15]

The PVN receives a particularly dense innervation of NPY-containing neurons, both from adrenergic and noradrenergic cells in the lower brainstem and from noncatecholaminergic cells in the arcuate nucleus.[69,91] The particular importance of the PVN and these projections in feeding is reflected in the finding that electrolytic lesions of the PVN significantly attenuate the feeding response elicited by intraventricular NPY injection,[92] similar to their effect on NE's action.[17] Moreover, the PVN is likely to be critical for the stimulatory effect of NPY on CORT release,[44] as well as for the feedback action of CORT on local α_2 and possibly NPY receptor sites.[2,17,81] As described below, the PVN, in addition to the arcuate nucleus, is distinguished by its changes in NPY content or NPY-positive innervation in response to food deprivation and subsequent refeeding.[93–95]

Biochemical Studies

Results of biochemical studies further support the proposal that NPY, while mimicking the action of exogenous NE, acts independently of presynaptic release of endogenous NE. Generally, in the periphery as well as the brain, NPY fails to enhance the turnover of NE and may, in fact, depress its release.[74,75,82,96] At low doses, NPY injection into the ventricles reduces NE utilization in the hypothalamus and specifically in the PVN.[97,98] Moreover, in freely moving animals with a microdialysis probe in the PVN, local injection of NPY fails to increase, and may tend to reduce, extracellular concentration of PVN NE.[99]

Alterations in hypothalamic NPY content, demonstrated in food-deprived, refed or genetically obese rats, also appear to differ from changes observed in levels or turnover

of NE in these animals. Food deprivation increases NPY content[93,95] or NPY-positive innervation[94] in the PVN, while it decreases the content and stimulates the turnover of NE in this nucleus.[100] Moreover, Zucker obese rats exhibit higher levels of PVN NPY content,[101] versus lower content of PVN NE.[102] Since only peptide levels were measured in these studies, the results with NPY are difficult to interpret, as the rise in PVN NPY content may actually reflect either an increase in transport from the arcuate nucleus or a decrease in NPY release and catabolism within the PVN.

Metabolic Studies

Investigations examining the effect of NPY on energy metabolism have further differentiated the effects of this peptide from those of NE. When injected into the PVN, NE has been shown to increase circulating glucose levels, whereas NPY appears to have no effect[44,103] (FIG. 4). Moreover, PVN NE also reduces energy expenditure, independent of its effect on locomotor activity,[47] whereas NPY has no effect on energy expenditure or activity level.[104] Instead, this peptide has a potent stimulatory influence on respiratory quotient, which increases in latency with increase in dose. This increase in respiratory quotient suggests that NPY potentiates fat synthesis preferentially from carbohydrate.

This effect on substrate utilization, a diversion of metabolism toward carbohydrate utilization and lipogenesis, may be related to the finding that acute NPY injection has a preferential stimulatory effect on carbohydrate ingestion[105] and that chronic NPY injec-

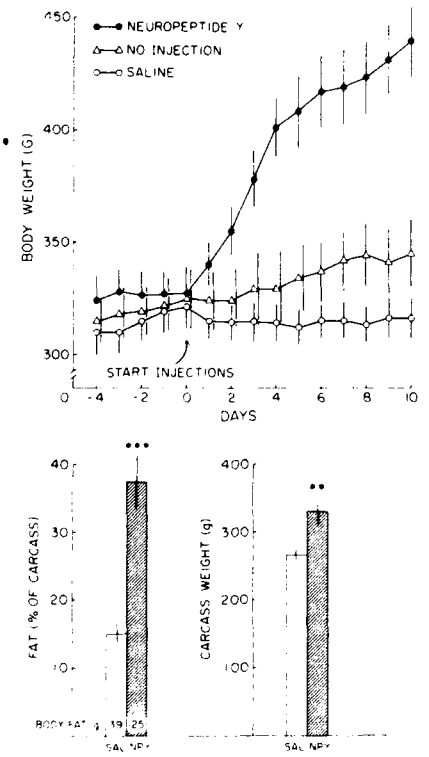

FIGURE 6. Measurements of body weight, carcass fat and carcass weight (mean ± SEM) in rats given chronic injections of PVN NPY or saline vehicle. $^{**}p < 0.01$, $^{***}p < 0.001$. (From Stanley *et al.*[106] Reprinted by permission from *Peptides*.)

tion has a potent effect on fat deposition and body weight and an increasing tendency to potentiate fat consumption[106,107] (FIG. 6). This pattern of effects observed with NPY may be distinguished from that of NE; that is, when chronically administered into the PVN, this amine selectively stimulates carbohydrate intake and has relatively little effect on fat ingestion, fat deposition and body weight gain.[30,32]

Physiological Function of Hypothalamic NPY in Relation to NE and 5-HT

As described above, there is strong evidence implicating the amines, NE and 5-HT, in the physiological control of food intake and appetite for carbohydrate, particularly at the onset of the active feeding cycle. The question is, does hypothalamic NPY also participate in this process, and, if so, to what extent does it interact with or depend upon the monoamines?

Evidence indicates that the relative importance of NPY as a cotransmitter increases with the frequency of the nerve impulses. That is, NPY coexists with NE primarily in large dense-cored vesicles, and intense, high frequency stimulation favors exocytosis of NPY from these large vesicles.[74,75,83] This suggests that NPY may exert its effect on food intake specifically under conditions that evoke high frequency stimulation, such as when energy stores are most depleted by an extended period of little eating.

Diurnal Rhythm of NPY and NE

It is proposed that NPY, in conjunction with NE or EPI, is activated specifically at the onset of the active feeding cycle to potentiate carbohydrate intake. The strongest evidence in support of this idea[2,38] is the finding that this particular time period is associated with: 1) a peak responsiveness to PVN injection of NPY; 2) a unimodal peak in PVN content of NPY; 3) a rise in the density of PVN α_2 receptors; and 4) an increase in the rat's natural preference for carbohydrate.[7,10,109] This is consistent with the report that NPY-elicited feeding is stronger in the dark cycle, as compared to the light cycle.[76] Furthermore, NPY injected into the PVN causes the release of CORT,[44] which is normally known to rise toward the onset of the dark cycle,[17,109] and the stimulatory action of this peptide on feeding is dependent upon high levels of circulating CORT.[2,81]

A state of energy depletion, and a consequent NPY release in response to intense neural stimulation, may similarly occur after environmentally imposed periods of deprivation. It is known that food deprivation stimulates carbohydrate intake as well as fat ingestion.[18] Deprivation, in addition to potentiating NE turnover and decreasing α_2-receptor sites specifically in the PVN,[110,111] has been shown to cause an increase in PVN NPY-positive innervation,[94] as well as a rise in NPY content.[93,95] A particular importance of NPY, relative to NE, in potentiating energy intake under such conditions may be suggested by its greater potency and longer duration of action.[72]

Temporal Characteristics and Potency of NPY and NE's Action

As described above, the latency, duration and potency of NPY's action are quite different from those exhibited by NE. The latency of these two responses differs considerably, with the amines acting within <1 min[8] and NPY after 10 min.[72] Moreover, their response duration also differs considerably, <30 min for NE and >4 hr for NPY. This difference in duration of action, which has similarly been detected in studies of the inhibitory effect of PVN NE and NPY on gastric acid secretion,[73] as well as in studies of

peripheral autonomic function,[74,75] allows NPY to yield a more potent effect. This long-term action and greater potency is particularly evident under conditions of *chronic* NPY stimulation of the PVN, which causes a marked increase in carbohydrate as well as fat ingestion, in association with a substantial rise in fat deposition and body weight[72,112] (FIG. 6). This is in marked contrast to the effects of chronic NE infusion, which has a more moderate effect on carbohydrate intake and produces little change in fat intake and body weight gain.[80]

Differential Metabolic Effects of NPY and NE

As described above, PVN injection of NE and NPY are found to have differential effects on energy metabolism, with NE reducing energy expenditure[47] and NPY increasing respiratory quotient and thus altering substrate utilization.[104] This difference between the metabolic, as well as body weight, effects of NPY and NE appears to qualitatively distinguish their mode of action and may also reflect quantitative differences in the latency and duration of their action.

Working Hypothesis

Based on this evidence, it is proposed that NPY has an important direct function as an auxiliary mediator of carbohydrate appetite at a time of greatest energy depletion. The working hypothesis is that NPY and NE, both exerting anabolic effects, function cooperatively and sequentially at the onset of the active cycle when carbohydrate plays a predominant role in restoring energy balance. Whereas NE acts rapidly to restore immediate energy requirements, NPY acts subsequently to produce lipogenesis and restore positive energy balance, particularly under conditions of greater food deprivation, energy deficits and sympathetic activation. With lower deprivation levels and energy requirements, NE at dark onset is initially activated, in association with CORT, to stimulate a carbohydrate meal, raise blood glucose and reduce energy expenditure. With greater energy deficits, sympathetic activation and consequent NE depletion, NPY, with its more potent and longer latency/duration of action, subsequently produces a stronger increase in carbohydrate feeding, releases insulin, increases respiratory quotient, and metabolizes carbohydrate to promote lipogenesis.

In addition to its interaction with NE, NPY may also interact with the monoamine 5-HT, which is believed to act specifically at the onset of the dark cycle, first, to inhibit carbohydrate intake and, then, to switch the animal's preference towards protein.[49] Neuropeptide Y is co-localized with 5-HT in dorsal raphe neurons known to innervate the hypothalamus and, in particular, the PVN.[116–118] Moreover, NPY is found to reduce 5-HT metabolism in the brain,[98,119,120] possibly via impact on presynaptic 5-HT_{1A} receptors,[121] and an antagonistic interaction between serotonergic stimulation and NPY-elicited feeding has been demonstrated.[122] Thus, NPY may potentiate ingestion of carbohydrate, in part, by controlling 5-HT release presynaptically and thereby disinhibiting the eating response.

Antagonistic Interaction between NPY and NE

While NPY and NE may initiate feeding via separate receptors on the postsynaptic membrane, there is additional evidence for a reciprocal antagonistic interaction between NPY and the α_2 receptors at the presynaptic terminal. This is reflected in the finding that

NPY via its own receptors acts similar to α_2 receptors in controlling the release of NE from noradrenergic nerve terminals (see above). In particular, NPY reduces NE utilization in the hypothalamus[98] and PVN,[97,99] and it has been shown to potentiate the presynaptic action of the α_2-receptor agonist clonidine in the brainstem.[113] A reciprocal release-modulating mechanism between the cotransmitters may also be operative, whereby NE via its presynaptic α_2 receptors controls the release of NPY, as well as the density of its receptors. This has been demonstrated by the finding that α_2-receptor antagonists, as well as NE or EPI synthesis inhibitors, enhance the release of NPY and potentiate its receptor action in the periphery and brain.[74,82,114,115]

Such an antagonistic interaction between NPY and NE specifically in the PVN may explain the finding that NPY is more effective in eliciting feeding when α_2 receptors are blocked and when endogenous NE stores are reduced or depleted. This result has been detected after PVN administration of NE synthesis inhibitors,[2,87] peripheral injection of α_2-receptor antagonists,[84] and brainstem knife cuts that damage NE or EPI fibers projecting to the PVN.[84]

In light of these diverse actions of NPY, it has been proposed that this peptide and NE may have a biphasic mode of interaction.[75] With specific reference to their physiological function in the PVN, it is suggested that NPY and NE have an initial cooperative, postsynaptic role in reinforcing the signal for carbohydrate ingestion at dark onset; subsequently, however, these cotransmitters interact presynaptically to antagonize the secretory mechanism of hyperactive nerve terminals and thus help to terminate the carbohydrate meal and thereby allow for ingestion of other macronutrients, such as protein. A shift of this nature, between carbohydrate and protein intake, has been found to occur naturally in freely feeding rats.[10,108]

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Regulation of MSH Secretion by Neuropeptide Y in Amphibians[a]

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Of all neuropeptides recently discovered, neuropeptide Y (NPY) has perhaps aroused the greatest interest. The isolation and characterization of the peptide from porcine brain extracts[1] was followed by a number of immunohistochemical studies to localize NPY-containing neurons in the central and peripheral nervous system of various mammalian species.[2-9] These studies have established the widespread distribution of NPY in the brain[2-6] and in sympathetic neurons of mammals.[6-9] The ubiquitous localization of NPY in central and peripheral nervous systems suggests that the peptide may exert pleiotropic activities. Consistent with this hypothesis, pharmacological studies indicate that, in mammals, NPY exhibits a number of potential functions including stimulation of feeding behaviour,[10] inhibition of sexual behaviour,[11] control of cardiovascular physiology[12-14] and modulation of neuroendocrine secretion.[15-17]

Now several studies have been conducted to examine the localization and characterization of NPY in various nonmammalian species including amphibians,[18-21] fish,[22,23] insects[24] and crustaceans.[25,26] In addition, neuroendocrine functions of NPY have also been investigated in these animals. In particular, NPY has been shown to modulate

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gonadotropin release in fish[27] and to inhibit α-MSH release[20,28,29] in the pars intermedia of anuran amphibians. This report summarizes data that describe the localization, characterization and neuroendocrine action of NPY in the brain and pituitary of amphibians. Part of these studies have been conducted in our laboratory and several contributions from other laboratories have also been incorporated into this report.

Localization of NPY in the Brain and Pituitary

The distribution of NPY-immunoreactive (IR) neurons has been described in detail in anuran and urodele amphibians, *e.g.*, the frogs *Rana ridibunda*[18] (FIG. 1) and *Rana catesbeiana*[21] and in the newt *Triturus carnifex*.[19] As in mammals, NPY-IR neurons appear to be widely distributed in the brain of these amphibian species.

Telencephalon: In amphibians, the dorsal and medial portions of the pallium show numerous perikarya and varicose fibers containing NPY.[18,19,21] A moderate number of NPY-IR cells is located in the pars medialis of the amygdala and in the posterior entopeduncular nucleus; scarce cells are visible in the nucleus of the diagonal band of broca.[18,19,21] The localization of NPY neurons in amphibians thus recalls the widespread distribution of NPY-IR fibers and perikarya in the cortex of mammals,[30,31] in particular in the entire hippocampal region of rat, monkey[32] and human.[33] The presence of NPY-neurons in the striatum and amygdala of the frog and newt is also consistent with the localization of NPY in the amygdaloid complex of rats[5] and in the human striatum.[34]

Diencephalon: NPY-IR cells are located in the posterocentral nucleus of the thalamus and in the ventromedial area of the thalamus.[18,19,21] The greatest accumulation of NPY-IR cell bodies and fibers in amphibians is observed in the medial basal hypothalamus.[18] The cell bodies are located in three distinct regions of the frog and newt hypothalamus:[18,19] i) the ventral infundibular nucleus, ii) the dorsal infundibular nucleus and iii) the suprachiasmatic and the posterior parts of the preoptic nucleus. The presence of a high density of NPY-containing neurons in the medial basal hypothalamus appears to be a common feature to mammals,[4,6,35] amphibians,[18,19,21] and fish.[22,23] However, some differences among species can be pointed out, such as the weak innervation of the suprachiasmatic nucleus observed in the cat brain[35] and the fact that, in the toad *Xenopus laevis*, NPY cell bodies are located only in the preoptic region of the hypothalamus.[20] In the frog[28] and toad brains,[20] a prominent bundle of NPY-containing fibers courses along the infundibulum and projects through the median eminence towards the pars intermedia of the pituitary. Thus, the pars intermedia of anurans receives a dense innervation (FIG. 2) which probably originates from the NPY cell bodies located in the medial basal hypothalamus. Similarly, in fish, the pituitary gland is directly innervated by NPY neurosecretory fibers.[22,23] In contrast, in the newt[36] and in mammals,[6] the pars intermedia is devoid of NPY-IR elements.

Mesencephalon: The highest density of NPY-IR perikarya in the mesencephalon of amphibians is located in the optic tectum.[18,19,21] Here, the perikarya are found in the periventricular area, central subdivisions and in the superficial grey layer (FIG. 3). In contrast, in the goldfish,[23] the NPY-IR cell bodies are restricted to the periventricular regions of the optic tectum. The superficial layers in both the fish[22,23] and amphibians[18,21] display a dense network of NPY-IR fibers. In the frog brain, NPY-IR

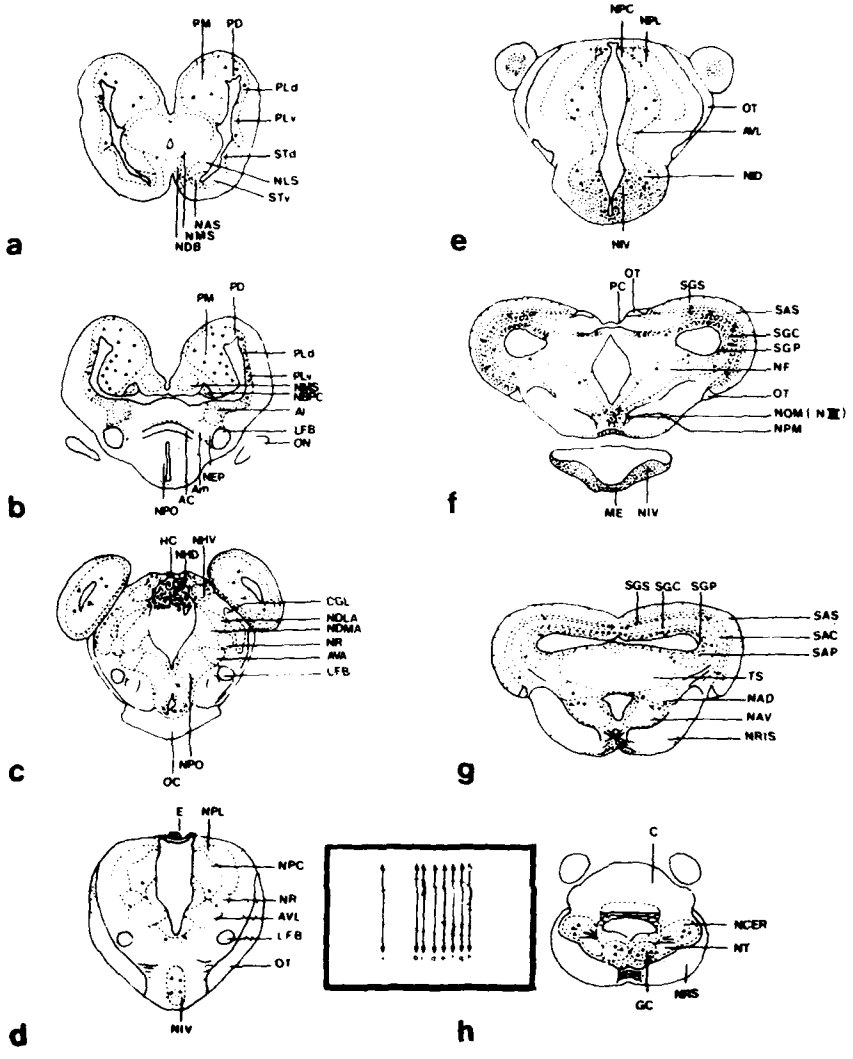

FIGURE 1. Schematic frontal sections through the brain of Rana ridibunda depicting the distribution of NPY-immunoreactive perikarya (*triangle*) and neuronal processes (*asterisks*). The density of fiber is meant to be proportional from region to region. The scheme of the sagittal section indicates the different planes of the frontal sections. AC: anterior commissure; Al: Amygdala, pars lateralis; Am: Amygdala, pars medialis; AVA: area ventralis anterior thalami; AVL: Area ventrolateralis thalami; CGL: Corpus geniculatus laterale; E: Epiphysis; GC: Griseum centrale rhombencephali; HC: Habenular commissure; LFB: Lateral forebrain bundle; ME: Median eminence; NAD: Nucleus anterodorsalis tegmenti mesencephali; NAS: Nucleus accumbens septi; NAV: Nucleus anteroventralis tegmenti mesencephali; NCER: Nucleus cerebelli; NDB: Nucleus diagonal band of Broca; NBPC: bed nucleus of the pollial commissure; NDLA: Nucleus dorsolateralis anterior thalami; NDMA: Nucleus dorsomedialis anterior thalami; NEP: Nucleus entopeduncularis; NF: Nucleus of the film; NHD: Nucleus habenularis dorsalis; NHV: Nucleus habenularis ventralis; NID: Nucleus infundibularis dorsalis; NIV: Nucleus infundibularis ventralis; NLS: Nucleus lateralis septi; NMS: Nucleus medialis septi; NOM (NIII): Nucleus of the occulomotor nerve; NPC: Nucleus posterocentralis thalami; NPL: Nucleus posterolateralis thalami; NPM: Nucleus profondus mesencephali; NPO: Nucleus preopticus; NR: Nucleus rotondus; NRS: Nucleus reticularis superior; NRIS: Nucleus reticularis isthmi; ON: Optic nerve; OT: Optic tract; PC: Posterior commissure; PD: Dorsal pallium; PLd: Dorsolateral pallium; PLv: Ventrolateral pallium; PM: Medial pallium; SAS: Stratum album superficiale tecti; SGC: Stratum griseum centrale tecti; SGP: Stratum griseum periventriculare tecti; SGS: Stratum griseum superficiale tecti; STd: Striatum, pars dorsalis; STv: Striatum, pars ventralis.

cells and fibers are also located in the dorsal and ventral parts of the ventral tegmentum and in the region just posterior to the torus semicircularis.[18] The interpeduncular nucleus of amphibians receives an NPY-innervation,[18,19,21] similar to the situation reported in mammals[4] and fish.[22,23]

Metencephalon: The cerebellum of amphibians is devoid of NPY-IR neurons.[18,19,21] The lack of NPY-innervation of the cerebellum is a constant feature within all the vertebrates species studied so far.[31] This is remarkable in view of the wide distribution of NPY in other brain areas.

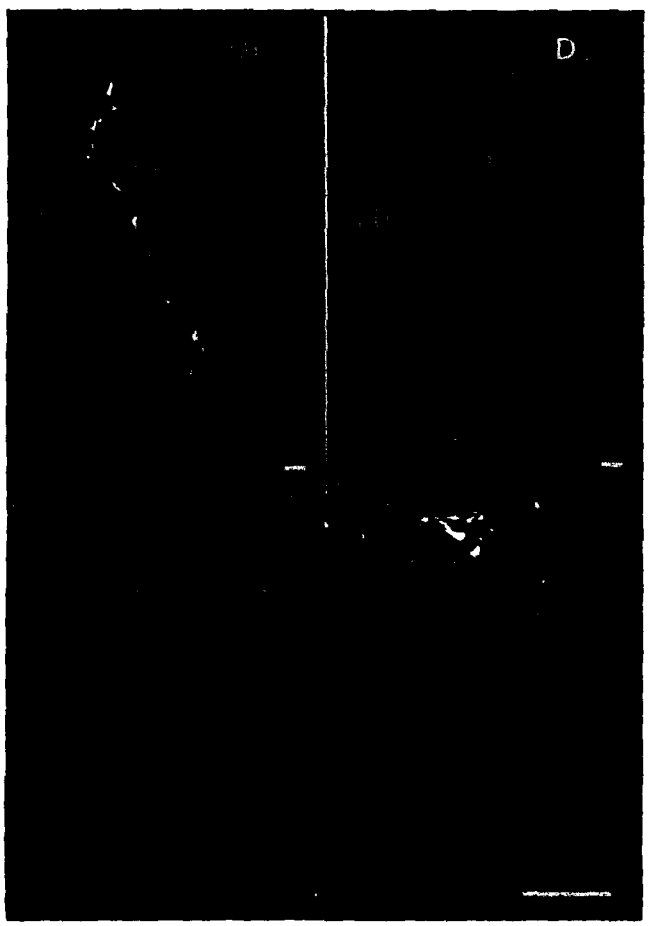

FIGURE 2. Immunohistochemical localization of NPY-containing fibers in the intermediate lobe of the frog pituitary. **(a)** General view of a sagittal section through the pituitary. **(b)** Control section adjacent to that shown in (a). Staining is completely prevented by prea-absorption of antiserum with 10^{-6} M synthetic NPY. **(c)** Higher magnification of a sagittal section of the intermediate lobe stained with anti-NPY serum. *White bar* represents 25 μm. DL: Distal lobe; IL: Intermediate lobe; NL: Neural lobe.

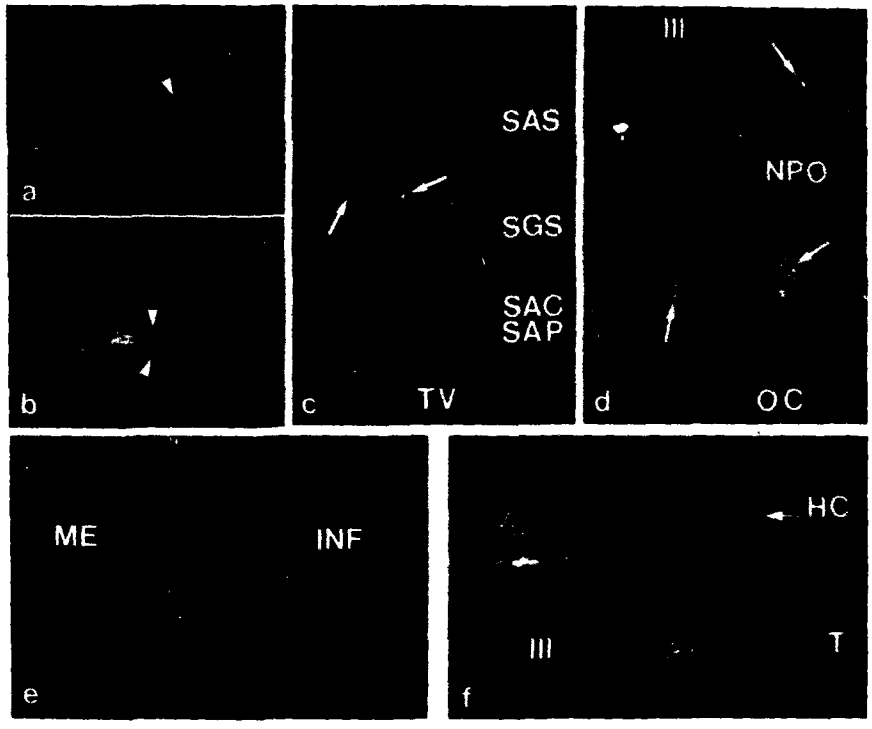

FIGURE 3. **(a)** Immunofluorescence photograph through the telencephalon showing typical monopolar NPY-positive perikaryon. (×620). **(b)** The nerve fibers (*arrow heads*) sometimes divided into two branches. (×820). **(c)** Frontal section through the optic tectum illustrating the zonation of immunoreactive fibers in the tectum. NPY-containing terminals are found in the superficial white matter (SAS). Two other layers of fibers are observed in the central white matter (SAC) and periventricular white matter (SAP). Perikarya (*arrows*) are visualized in the superficial gray matter (SGS). (×140). TV: Tectum ventricle. **(d)** Frontal section through the suprachiasmatic region of the preoptic nucleus (NPO). Groups of perikarya are found in the ventral periventricular region. Scattered cell bodies are also observed more dorsally throughout the suprachiasmatic nucleus. (×145). OC: Optic chiasma; III: Third ventricle. **(e)** Sagittal section of the median eminence (ME). Immunoreactive fibers issuing from the infundibulum (INF) are mainly distributed in the external zone. (×210). **(f)** Frontal section at the level of the habenular region showing symmetric groups of fibers surrounding the habenular nucleus. A positive tract of discrete fibers is observed in the habenular commissure (HC). The telencephalon (T) contains also numerous NPY-IR fibers. (×71).

Rhombencephalon: In the frog brain, two dense tracts of NPY-IR fibers are running ventrally and dorsally in the medulla oblongata. However, no cell bodies are present in this brain region.[18]

Several immunocytochemical studies have been conducted at the electron microscopic level to determine the subcellular localization of NPY in lower vertebrate species.[18,28] In particular, staining of ultrathin sections with NPY antiserum has made it possible to identify positive neurons in the infundibulum[18] and the pars intermedia of the frog.[28] The NPY-IR material appears to be concentrated in dense core vesicles of about 100 nm in diameter. In the infundibulum, about 10% of the NPY-containing fibers display synaptic

contacts with other nerve fibers.[18] In the intermediate lobe of the frog pituitary,[28] NPY-positive nerve fibers are found coursing among the endocrine cells of the intermediate lobe (FIG. 4).

Co-Localization of NPY and the C-Terminal Flanking Peptide of Neuropeptide Y

Using molecular biology approaches, the structures of the rat[37] and human[38] precursor molecules to NPY have been established. NPY is synthesized as a 97-amino-acid pre-pro-peptide. Proteolytic cleavage of the precursor protein gives rise to NPY, and a 30-amino-acid cryptic peptide which has been designated as "the *c*-terminal flanking *p*eptide *o*f *n*europeptide Y (C-PON)."[39] The distribution of C-PON-IR neurons has been studied in the frog[21] using an antibody raised against the human C-PON sequence.[39] NPY and C-PON exhibit a remarkable co-distribution in *Rana ridibunda*[40] and *Rana catesbeiana*.[21] The two peptides are co-located within the same cell bodies throughout the brain (FIG. 5) and sequential double immunohistochemical staining methods[41] indicate that NPY and C-PON are also co-localized within the same nerve processes.[40] In the intermediate lobe of the pituitary, numerous fibers containing both NPY- and C-PON-IR have been observed[40] (FIG. 6). These data suggest that the amphibian C-PON peptide is similar to the deduced mammalian sequences and therefore show that the structure of the propeptide has been highly preserved during evolution. The fact that C-PON is also present in the pars intermedia of the frog pituitary indicates that the peptide is transported distally in nerve terminals and is likely co-released with NPY.

FIGURE 4. Immunoelectron microscopic localization of NPY in the intermediate lobe. Immunostaining is restricted within a nerve fiber coursing between parenchymal cells. The gold particles are located in dense core vesicles. (× 40,000).

Biochemical Characterization

NPY-IR material in amphibian species has also been studied using a highly specific radioimmunoassay method.[18] Serial dilutions of frog brain and neurointermediate lobe extracts give binding curves which parallel that of synthetic porcine NPY.[18,28] This indicates that the amphibian peptide and porcine NPY have a close structural relationship. The highest concentration of NPY is present in the neurointermediate lobe of the pituitary (TABLE 1). The infundibulum and the preoptic region of the hypothalamus of the frog[18] and the newt[19] also contain large quantities of NPY-like material. Gel permeation chro-

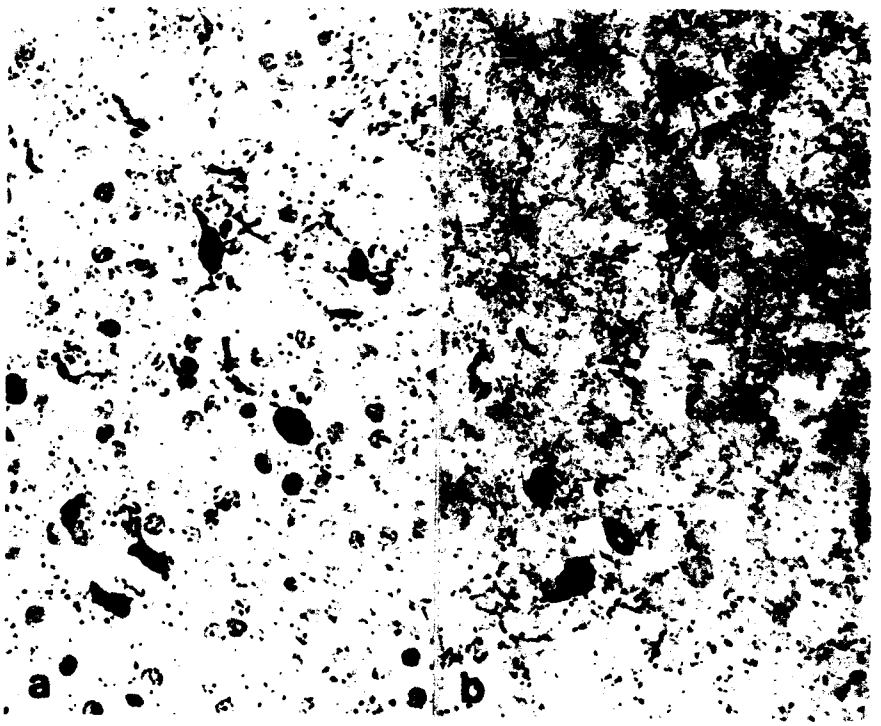

FIGURE 5. Serial frontal sections through the dorsal pallium. Using the PAP technique, the same perikarya stain for NPY (**a**) and C-PON (**b**). (×215).

matography resolves a major peak of NPY-IR material which corresponds to a compound having an apparent molecular weight similar to that of synthetic NPY.[18] SDS-polyacrylamide gel electrophoresis, followed by immunoblotting detection of NPY-IR indicates that the newt peptide co-migrates with mouse NPY-IR and with synthetic porcine NPY. It exhibits an apparent molecular weight of 4,000 daltons.[19] A minor peak corresponding to a high molecular weight immunoreactive form of NPY is also resolved by gel permeation of frog and newt brain extracts.[18,19] This likely reflects the presence of unprocessed precursor in the frog brain. Analysis by reverse phase high performance liquid chromatography (HPLC) of neurointermediate lobe extracts clearly indicates that the retention

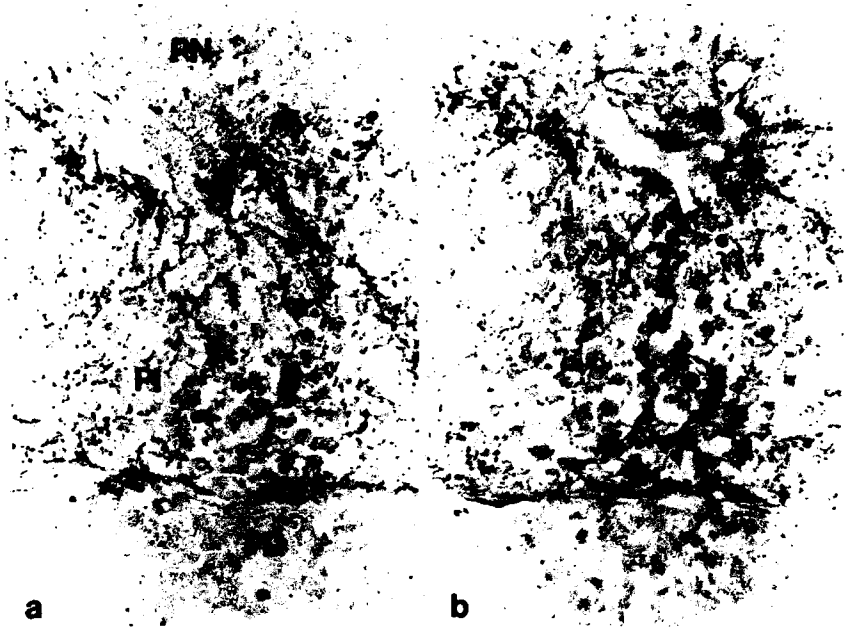

FIGURE 6. Serial frontal sections through the pituitary. Using the PAP technique antisera to NPY (**a**) and C-PON (**b**) stained a network of fibers in the pars intermedia (PI). (× 188). PD: Pars distalis; PN: Pars nervosa.

time of frog NPY-IR peptide is shorter than that of synthetic porcine NPY,[18,28] thus indicating that the former peptide is less hydrophobic (FIG. 7). The newt NPY-like peptide appears to be, as well, less hydrophobic than porcine NPY;[19] in fact, human[42] and bovine NPY[43] also exhibit a shorter retention time than porcine NPY. Comparison of the structure of the human peptide with the porcine molecule shows that leucine 17 in the latter peptide has been substituted by a methionine in the former peptide. This substitution accounts for the difference in the chromatographic behaviour of these peptides. Minor changes in the primary structure of the amphibian molecules relative to that of porcine NPY will probably explain the shorter retention times of these peptides.

TABLE 1. Immunoreactive NPY Contents and Concentrations in the Frog Brain and Pituitary[a]

Brain Region	NPY Concentration (pmole/mg Protein)
Neurointermediate lobe (n = 8)	693 ± 88
Infundibulum (n = 8)	233 ± 16
Preoptic region (n = 8)	151 ± 12
Telencephalon (n = 8)	163 ± 8
Mesencephalon and thalamus (n = 8)	60 ± 13
Medulla oblongata (n = 8)	48 ± 15
Cerebellum (n = 8)	ND

[a]Values are given as mean ± SEM; the number of animals in each group is shown in parentheses. ND, non detectable.

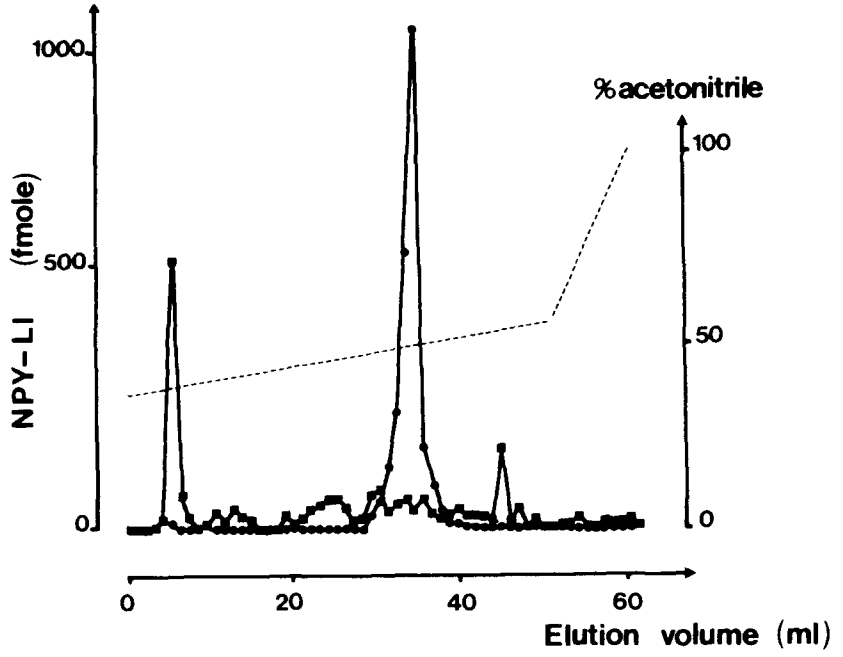

FIGURE 7. Reverse phase HPLC analysis of frog neurointermediate lobe extracts. The gradient used is shown on the figure. ■-■: neurointermediate lobe extracts. ●-●: synthetic NPY.

Control of Alpha-MSH Secretion

On the basis of anatomical data indicating the existence of a dense innervation of the pars intermedia of anuran amphibians by NPY-containing fibers, a possible role of NPY in the control of pars intermedia was investigated in these species. The first demonstration that NPY is actually involved in the regulation of melanotropin secretion was provided in the frog *Rana ridibunda*.[28] This study was conducted by means of the *in vitro* perifusion technique.[44] Through the use of this dynamic incubation system, coupled to a highly specific radioimmunoassay,[45] to monitor the release of α-MSH from the pars intermedia, the kinetics of secretory response of melanotropes to various exogenous secretagogues can be analyzed. Using this model, it has been demonstrated that administration of graded concentrations of synthetic porcine NPY induces a dose-dependent inhibition of α-MSH from neurointermediate lobes of *Rana ridibunda* (FIG. 8). Half-maximum inhibition is observed with a dose of 5×10^{-8} M; no desensitization occurs during prolonged infusion of NPY.[46] The inhibitory action of NPY on α-MSH release has subsequently been confirmed in two other amphibian species, the toad *Xenopus laevis*[20] and the frog *Rana pipiens*.[47] By means of pulse-chase experiments it has also been demonstrated, in the toad, that NPY inhibits coordinately the release of all proopiomelanocortin (POMC)-derived peptides.[20] In the sort term, at least, NPY is without effect on the biosynthesis or processing of the precursor POMC.[20] *In vivo* experiments showed that administration of NPY to freely moving Xenopus causes a dramatic aggregation of melanophores in black background-adapted animals (unpublished observation), suggesting that NPY plays a physiological role in skin color adaptation. In the newt *Triturus cristatus*, which lacks NPY innervation of the pars intermedia, NPY has no effect on α-MSH secretion.[36] A

comparative study has shown that while NPY inhibits α-MSH secretion from the pars intermedia of anurans, it has no effect on α-MSH release in the rat.[47] This is consistent with the lack of NPY innervation[6] and binding sites[48] in the pars intermedia of the rodents. Symmetrically, in the goldfish, the intermediate lobe is richly innervated by NPY fibers[7] and in this species, NPY modulates α-MSH secretion.[49]

The structure-activity relationship of NPY, with respect to its inhibitory effect on α-MSH secretion from the frog pars intermedia, has been investigated.[29] In this study, the relative potencies of various natural and synthetic peptides chemically related to NPY were compared (FIG. 9). The most potent peptide in inhibiting α-MSH release is NPY. The synthetic analogue NPY [2–36] also induces a dose-related inhibition of α-MSH secretion and the dose-response curves for both NPY and NPY [2–39] are parallel. The fragments NPY [16–36] and NPY [25–36] are much less active and provoke a slight inhibition only at high concentrations. The fragment NPY [1–15] has no effect on α-MSH secretion. The order of inhibitory potency of the various NPY analogues is: NPY > NPY [2–36] > NPY [16–36] > NPY [25–36] > NPY [1–15]. These observations are in good agreement with the results of the structure-activity studies on NPY analogues conducted with various mammalian tissues.[50–52] The fact that the fragments NPY [16–36] and NPY [25–36], despite a considerable loss of activity, still exhibit α-MSH release inhibitory activity, suggests that the biological determinant of NPY is contained in the C-terminal region of the molecule.

Two pancreatic peptides have been tested for inhibitory activity on the amphibian pars intermedia, namely peptide YY (PYY) and avian pancreatic polypeptide (APP). Of these, PYY appears to be the most active but the dose-response curve is not parallel to that obtained with NPY.[29] This result is consistent with recent receptor binding experiments which show that PYY actually binds the NPY receptor in the brain of rodents.[48,49] However, whether PYY can be considered to be physiologically involved in the regulation of α-MSH secretion is doubtful since this peptide is not located in the central nervous system[53] and consequently not contained in nerve fibers of the pars intermedia. The other member of the pancreatic polypeptide family, avian pancreatic polypeptide (APP), also induces an inhibition of α-MSH release.[29] However, the effect is weak and is observed

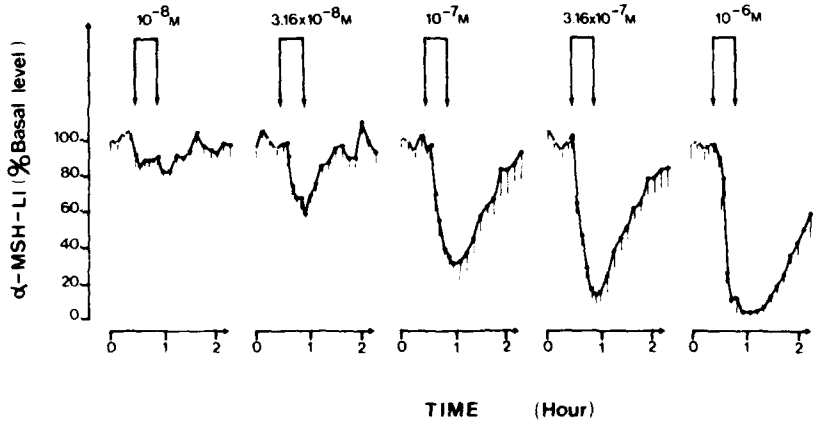

FIGURE 8. α-MSH secretion by isolated perifused frog neurointermediate lobes in response to graded doses of synthetic NPY. The reference level (100% basal release) was calculated as the mean α-MSH secretion rate during 30 min (4 consecutive fractions; ○-○) just preceding the infusion of NPY.

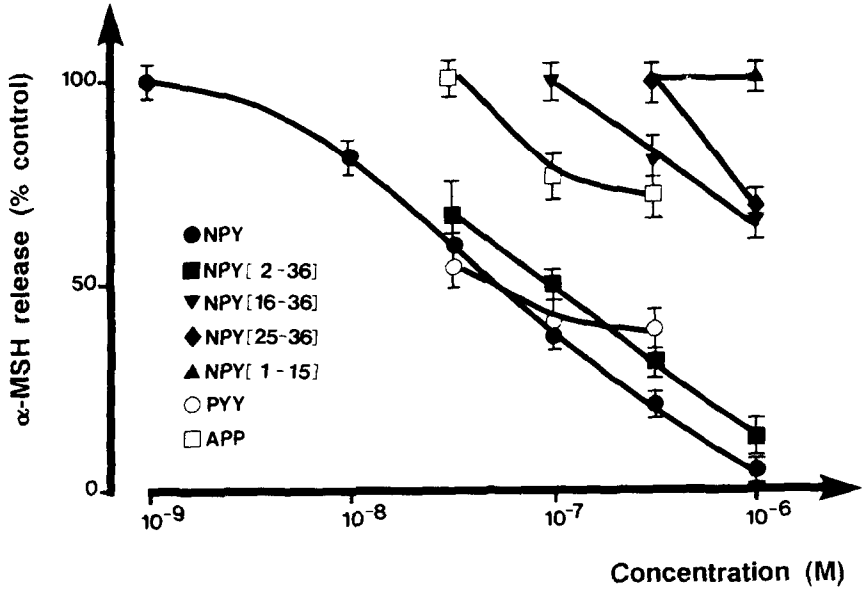

FIGURE 9. Semi-logarithmic plot comparing the effects of NPY, various NPY short chain analogues, PYY and APP on α-MSH release by perifused frog neurointermediate lobes. The mean α-MSH concentration in the fractions collection just before the end of infusion of NPY or related peptides (maximum inhibition) was compared to the mean α-MSH level measured just prior the infusion of each dose of the peptide (spontaneous secretion; 4 consecutive fractions). Each point represents the mean (±SEM) of 3 independent perifusion experiments.

only at high concentrations. A comparison of the structure of the three pancreatic peptide-related molecules reveals the likely basis for their biological potencies. PYY has a high degree of homology with NPY in the 16–36 region of the molecule (71% homology), whereas APP, which is far less potent than NPY and PYY, exhibits 5 amino acid substitutions when compared to NPY and PYY (Tyr 20, Arg 25, His 26, Leu 30, His 34). These data suggest that one or several of these C-terminal residues are essential for the bioactivity of the peptides. Since removal of the C-terminal amide group has been shown to drastically reduce the biological activity of most amidated peptides, the tyrosyl-amide residue of NPY may also be of crucial importance for the bioactivity of NPY. All these observations give support to the concept that the NPY receptors have been highly preserved during evolution.

Mechanism of Action

Two classical neurotransmitters, dopamine[54] and GABA,[55,56] are also potent inhibitors of melanotropic cell activity. In order to determine whether NPY acts presynaptically on dopamine- or GABA-containing fibers, the effect of NPY has been investigated during prolonged administration of haloperidol and bicuculline. The inhibitory effect of NPY in the frog is not affected by the dopaminergic (FIG. 10) and GABAergic antagonists.[28,31] These data, together with the results of ultrastructural studies (see above) suggest a direct action of NPY on melanotropes. This has been more directly shown using a perifusion

model with dispersed frog pars intermedia cells, a preparation in which all afferent fibers are destroyed. It was found that NPY induces inhibition of α-MSH release in frog.[46] Thus, in this animal, NPY exerts a direct action on melanotropes. This observation is also given support by results of recent electrophysiological experiments, carried out on a primary culture of frog pars intermedia cells, using the patch-clamp technique. NPY induces hyperpolarization in these cells and inhibits spontaneous action potentials.[57] With respect to the mechanism of action of NPY, some species differences may be expected. In contrast to the frog, in the pars intermedia of the toad *Xenopus laevis*, the melanotropes may not constitute the main target for NPY, since it has no effect on dispersed pars cells in this animal (B.G.J., unpublished data).

The pars intermedia of the frog pituitary is directly innervated by thyrotropin-releasing

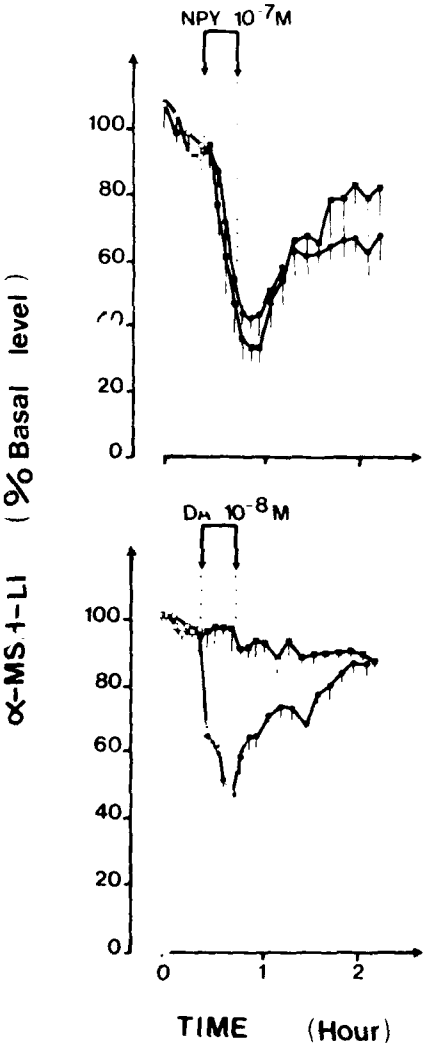

FIGURE 10. Differential effects of haloperidol upon the inhibitory actions of NPY (10^{-7} M) and dopamine (10^{-8} M) on α-MSH secretion. Frog neurointermediate lobes were continuously perifused in the absence (●) or in the presence (■) of haloperidol (10^{-5} M).

hormone (TRH)-IR fibers[58] and we have previously shown that TRH is a potent stimulator of α-MSH secretion in anuran amphibians.[59,60] Since the intermediate lobe of the pituitary of anurans, unlike urodeles,[36] is under the antagonistic control of TRH and NPY, possible interactions between these two neuropeptides on α-MSH secretion have been examined *in vitro*. During prolonged administration of NPY, the stimulatory effect of TRH is inhibited in a dose-dependent manner.[46] Half-maximum inhibitions of α-MSH release (both basal and TRH-induced secretion) are observed at a NPY concentration of 3×10^{-8} M. These data suggest that NPY and TRH act through a common intracellular pathway to regulate α-MSH secretion in the frog. Since it has been shown recently that TRH produces an increase of phosphoinositol breakdown in the frog pars intermedia,[61] a possible effect of NPY on this second messenger system has been examined: no effect of NPY on the level of inositols could be demonstrated (unpublished results). A possible action of NPY on adenylate cyclase synthesis is currently being examined. Primarily, results indicate that NPY induces a dose-dependent inhibition of the synthesis of cyclic-adenosyl monophosphate (c-AMP).

In conclusion, an authentic NPY is present in the brain of amphibians and the peptide located in the pars intermedia of the pituitary of anurans can be considered has a physiological melanotropin-inhibiting factor. Since the pars intermedia is composed of a homogeneous population of endocrine cells, this tissue appears well-suited for investigating the intra-cellular mechanisms which mediate the action of NPY.

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Regulation of the Hypothalamo-Pituitary Axis by Neuropeptide Y

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Immunohistochemical studies have demonstrated neuropeptide-Y (NPY) immunoreactivity in neuron cell bodies in the arcuate, periventricular, anterior, supraoptic and paraventricular (PVN) nuclei of the hypothalamus.[1-3] In addition, in situ hybridization histochemistry has similarly shown the presence of NPY mRNA in many of these areas.[4] Dense NPY fiber networks have been found throughout the hypothalamus, with particularly dense concentrations of fibers in the arcuate, PVN, periventricular and preoptic nuclei.[1-3] Furthermore, it appears that the NPY terminals in the PVN may be derived from several sources. The hypothalamic arcuate nucleus[5,6] and brainstem NPY-containing cell bodies project to the PVN.[7-9] The innervation of the PVN by brainstem NPY fibers may also contain epinephrine or norepinephrine.[7-9] However, lesioning the catecholaminergic neurons with 6-hydroxydopamine has resulted in the sparing of hypothalamic NPY neurons in spite of dramatic depletion in hypothalamic norepinephrine concentrations,[10,11] raising doubts about the origin of NPY in the PVN. Although, Bai *et al.* have suggested a greater contribution by the arcuate NPY cell bodies to the PVN.[5] The NPY projections to the PVN also appear to be directed toward the parvocellular portion of the nucleus and directly synapse on neurons containing corticotropin-releasing hormone (CRH).[12] In addition, projections also are directed at magnocellular neurons of the supraoptic nucleus containing vasopressin.[13]

The physiological function of hypothalamic NPY has yet to be determined although data from the laboratories of McDonald *et al.* and Kalra *et al.* suggest that NPY may act to enhance reproductive function in the female rat and may be involved in coordinating the release of luteinizing hormone during the estrous cycle.[14,15] In addition, convincing data from the laboratories of Leibowitz *et al.* and Kalra *et al.* suggest that NPY, especially in the PVN, is responsible for stimulating food intake in the rat.[16,17] NPY may also be involved in regulating fluid homeostasis in the rat.[18]

Several groups have convincingly demonstrated that NPY secretion is linked to regulation of metabolic hormone secretion, in particular, the regulation of ACTH secretion. Härfstrand *et al.*[19] initially reported the ability of NPY to augment the secretion of ACTH and corticosterone in the rat following infusion into the ventricular system of the rat brain.[19] Subsequently, Wahlestedt *et al.*[20] and Haas and George[21] confirmed this finding. In addition, Haas and George showed that NPY administration increased the concentration of corticotrophin-releasing hormone in the hypothalamus, suggesting the induction of CRH synthesis by NPY. NPY-induced activation is thought to be mediated by the enhanced secretion of CRH by the hypothalamus.[22] NPY also has been found in the hypophysial portal circulation[23] and in the anterior pituitary.[24] As a consequence, NPY may mediate its actions at multiple sites in the hypothalamic-pituitary adrenal axis.

These pharmacological and anatomical studies suggest that NPY is potentially capable of modulating ACTH secretion physiologically, especially during responses to stress when multiple factors are activated to insure a robust ACTH secretory response. The

present studies were designed to further characterize the role of hypothalamic NPY in mediating ACTH responses to physiological challenges in the rat.

EXPERIMENTAL METHODS

Adult male Sprague-Dawley rats (175–200 gms) were purchased from Charles River Inc. (Kingston, NY). The animals were housed in a temperature-controlled animal room on a fixed light cycle (lights on 0700–1900 hrs). The animals had free access to rat chow and water at all times.

Hypophysial Portal Blood Collection

Hypophysial portal blood was collected according to the protocol of Porter and Smith.[25] Briefly, the rats were anesthetized with urethane (1.2 gm/kg). The ventral surface of the skull was exposed by the parapharyngeal approach and the bone over the pituitary gland was carefully removed. The dura in the floor of the sella was cut and the pituitary gland was reflected forward to expose the pituitary stalk. The pituitary stalk was transected and the gland removed. Heparinized saline (0.5 ml) was administered through an intravenous cannula. A polyethylene cannula was placed over the stump of the cut stalk and blood was collected by gravity into a chilled tube containing aprotinin (Sigma). Collections were of 30–60 min duration. Following centrifugation, the plasma was stored at −80°C.

Dispersed Pituitary Cell Cultures

Male rats weighing 175–200 g were sacrificed by decapitation. The anterior lobe of the pituitary gland was isolated and dispersed monolayer cell cultures were prepared as previously described[26] with several modifications. Anterior lobes of the pituitary were mechanically disrupted and incubated with collagenase, DNase and pancreatin as described by Vale *et al.*[27] The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (2%), T_3 (60 pm), insulin (5 μg/ml), transferrin (5 μg/ml) and FGF (1 ng/ml) and plated in plastic multiwell plates. The cultures were incubated in a humid atmosphere containing 5% CO_2 for 3 days at which time the cells were washed 3 times with DMEM and were incubated for an additional 24 hours in serum-free test medium which consists of DMEM and a serum substitute (University of California, San Francisco, Cell Culture Facility). The following day the cells were washed 4 times with DMEM and groups of 6 wells were incubated for 3 hours with the peptides to be tested. At the end of the incubation, the medium was removed from the cells with a polypropylene pipet and transferred directly into chilled polypropylene assay tubes. Multiple aliquots were prepared to prevent freezing and thawing artifacts. Medium concentrations of ACTH were determined by radioimmunoassay (see below).

Tissue Processing

The brain and pituitary were rapidly removed from the skull. The neurointermediate lobe of the pituitary (NIL) was separated from the anterior lobe and placed in 200 μl of 2 N acetic acid. The brain was placed on wet ice and the median eminence (ME) was

removed with the aid of a dissecting microscope and fine iris scissors. After removing the ME, the brain was frozen on dry ice for later dissection. The ME was placed in 200 μl of 2 N acetic acid. Both the ME and NIL were placed in a boiling water bath for 20 min. After boiling, the extracts were aliquoted and lyophilized. The frozen brain was dissected into an anterior hypothalamic fragment (POA) containing the preoptic area, paraventricular, suprachiasmatic and supraoptic nuclei and a mediobasal hypothalamic (MBH) fragment containing the arcuate, ventromedial and dorsomedial nuclei. These fragments were placed in 1 ml of 2 N acetic acid and heated in a boiling bath for 20 min. The tissue debris was pelleted by centrifugation and the extracts were aliquoted and lyophilized. The tissue pellets were dissolved in 1N NaOH for protein determinations.

Radioimmunoassays

Plasma concentrations of ACTH were determined using standard procedures.[28] The ACTH antiserum was purchased from IgG Corp. (Nashville, TN) and radioiodinated ACTH was purchased from Radioassay Systems Laboratory (Carson, CA). Porcine ACTH purchased from IgG Corp. (Nashville, TN) was used as standard. The assay sensitivity was 1 pg/ml. The intra- and interassay coefficients of variation were 6.4% and 9.8%, respectively.

Tissue concentrations of NPY were determined by radioimmunoassay as described by Allen *et al.*[29] Plasma samples were processed as previously described.[23] Briefly, antisera to NPY were raised in New Zealand white rabbits against porcine NPY conjugated to bovine serum albumin with carbodiimide. ^{125}I-NPY was purchased from New England Nuclear (Boston, MA). The assay was run under nonequilibrium conditions; antibody was incubated with the samples for 72 h in the absence of tracer. Following a further 72-h incubation in the presence of tracer, free ^{125}I-NPY was separated from bound by addition of goat-anti-rabbit serum. Assay sensitivity was 2 pg/tube and inter- and intraassay coefficients of variation were 8.8% and 6.6%, respectively.

Protein Determinations and Statistical Analysis

The concentration of protein in the tissue extracts was determined using the fluorometric method as described by Bohlen *et al.*[30]

Statistical differences were determined by analysis of variance and Student t test according to procedures outlined by Zar.[31] Values of p less than 0.05 were considered significant.

RESULTS

Hypophysial Portal Blood Concentrations of NPY

NPY immunoreactive material was readily detectable in the hypophysial portal blood of the urethane-anesthetized male rat. Portal plasma concentrations of NPY in the first 30 min collection period were 52.0 ± 4.0 ng/ml. Continuing the collection for an additional 30 min did not significantly alter NPY levels. Furthermore, a significant portal-systemic gradient existed for NPY as systemic plasma concentrations of NPY were 16.0 ± 4.5 ng/ml (p <0.004). These levels were not significantly altered during a second 30-min period.

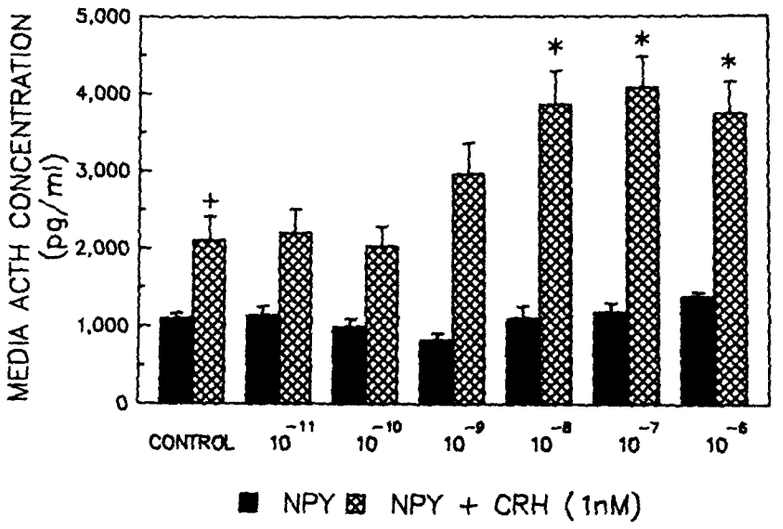

FIGURE 1. The effect of NPY on ACTH secretion from dispersed anterior pituitary cells in the absence or presence of 1 nM CRH. Anterior pituitary cells were enzymatically dispersed and plated at a density of 250,000 cells per well. The cells were incubated with NPY (Richelieu Biotechnologies), CRH (1 nM; Peninsula Laboratories) or the combination of NPY and CRH for 3 hours. The medium was collected in chilled plastic tubes and frozen immediately. Shown in the figure is the group mean from 6–12 wells per point and the standard error of the mean. *-indicates statistical significance at the 0.05 level compared to the appropriate control.

Actions of NPY on ACTH Secretion from Dispersed Anterior Pituitary Cells

Previous studies from several laboratories demonstrated the dose-dependent stimulatory action of NPY on the secretion of LH, FSH and GH from perifused dispersed anterior pituitary cells.[32,33] However, an effect of NPY on ACTH secretion was not observed.[33] FIGURE 1 depicts the effect of NPY on ACTH secretion from dispersed anterior pituitary cells derived from male rats.

Anterior pituitary cells were plated at a density of 250,000 cells per well. The addition of NPY (10^{-11} M to 10^{-6} M) was without effect on the release of ACTH into the media during a 3-hr incubation period. Whereas, the addition of 1 nM CRH to the wells significantly stimulated ACTH secretion ($p<0.001$) under basal conditions. The coincubation of CRH (1 nM) and NPY (10^{-11} to 10^{-6} M) resulted in a dose-related augmentation of the stimulatory effect of CRH ($p<0.005$). Maximal secretion of ACTH occurred when 1 nM CRH and 100 nM NPY were coincubated. This combination resulted in a 100% increase in ACTH secretion compared to CRH alone.

Interaction between NPY and ACTH Regulation during Stress

Stress of various types has been found to increase the activity of the hypothalamo-pituitary-adrenal axis as reflected in plasma concentrations of ACTH or corticosterone. Hypovolemic stress has been associated with an activation of CRH secretion into the

hypophysial portal circulation,[34] whereas insulin hypoglycemia-induced activation of ACTH secretion is apparently mediated by activation of vasopressin secretion.[35] Although CRH mechanisms may also be important in this response.[36,37] Recent studies also have identified CRH as the mediator of interleukin-induced ACTH secretion.[38,39] However, the location of NPY in the long projection neurons derived from brainstem NE sources implicate this neuropeptide as a potential mediator of these responses.

Activation of immune mechanisms have been associated with a concomitant increase in ACTH or corticosterone secretion.[38,39] In the present study, FIGURE 2 depicts the time course of the endotoxin-induced release of ACTH. Plasma concentrations of ACTH peak 3–4 hrs after the injection of bacterial lipopolysacchride (25 μg/kg, i.p.). At the time when ACTH levels are at their peak, concentrations of NPY in the anterior hypothalamus/POA were increased 33% ($p < 0.001$; FIG. 3). However, concentrations of NPY in other brain regions and the pituitary gland were unchanged.

In contrast to the minimal changes observed in NPY concentrations during circadian ACTH secretion or LPS-induced stress, insulin hypoglycemia induced a much greater and more rapid increase in plasma ACTH concentrations (FIG. 4). ACTH concentrations increased approximately 25-fold 30 min after the injection of human recombinant insulin (5 IU/kg, i.p.). The sharp decrease in plasma glucose concentrations resulted in a 60% depletion in POA NPY concentrations and a 40% reduction in the basal hypothalamus (MBH) (FIG. 5). In contrast to these regions, a highly significant increase in NPY content was observed in the neurointermediate and anterior lobes of the pituitary gland (FIG. 6). Ninety minutes after insulin infusion, ACTH levels were still greater than 400 pg/ml but were decreasing. The depletion of NPY in the POA fragments observed at 30 min was still evident at this time; however, NPY concentrations in the basal hypothalamus were increased 50% over basal levels and 200% over the 30-min time point. In addition, the concentration of NPY in the ME increased 100% over basal levels. NPY concentrations in both lobes of the pituitary returned to pretreatment levels.

DISCUSSION

Previous studies have indicated that the infusion of pharmacological amounts of NPY activated the hypothalamo-pituitary-adrenal axis of the rat.[19–21] Furthermore, these stud-

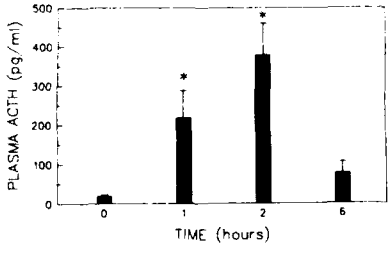

FIGURE 2. The effect of endotoxin on plasma concentrations of ACTH. Male Sprague-Dawley rats were treated with bacterial lipopolysacchride (25 μg/kg, i.p.; serotype 05:55, Sigma). The animals were sacrificed by decapitation 1 hr after vehicle treatment or 1, 2, 4, 6, and 24 hours after injection of endotoxin. Brains were rapidly removed, the median eminence was dissected from the fresh brain and peptides were extracted in boiling acetic acid. The remainder of the brain was frozen for later dissection. The frozen brain was dissected into POA and MBH fragments, and peptides were extracted in boiling acetic acid. Blood samples were collected in ice-cold tubes containing EDTA at the time of sacrifice. Plasma was separated by centrifugation and aliquoted for the ACTH assay. Each bar represents the mean ACTH response and the standard error of the mean in groups of 8 animals. *-indicates significant differences at the 0.05 level vs the control animals treated with vehicle 1 hr before.

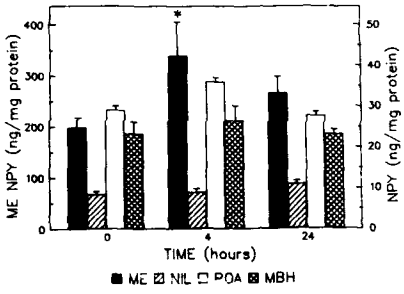

FIGURE 3. The effect of endotoxin on NPY concentrations in several brain regions 4 and 24 hours after treatment. NPY concentrations for the ME are depicted on the *left axis* whereas other regions should be compared to the *right axis*. See FIGURE 2 legend for further details.

ies implicated a central nervous site of action for NPY. In the present studies, we have identified the presence of NPY in the hypothalamo-pituitary portal circulation. The concentration of NPY in the portal blood was 3–5 times greater than the amount in the systemic circulation suggesting active secretion of NPY from nerve terminals in the median eminence.[23] Recent studies have indicated that NPY also may be secreted by the anterior pituitary.[24] However, the pituitary gland was removed from the animals in advance of the collection of the portal blood, so that the source of NPY was restricted to systemic sources or the median eminence.

Our studies also have demonstrated the ability of NPY to potentiate CRH-induced ACTH secretion under in vitro conditions. NPY alone was unable to act as an ACTH secretogogue in doses up to 10^{-6} M, similar to the results obtained by Kerkerian *et al.*[33] The CRH potentiating activity of NPY was dose-related with maximal actions at 10^{-8} to 10^{-7} M. These concentrations of NPY are similar to the concentration of the peptide detected in the hypophysial-portal blood studies suggesting that NPY may function as an endocrine or paracrine factor modulating ACTH secretion in the pituitary. In addition, the data of Bloom *et al.*[24] enhance the likelihood of a paracrine function for NPY in the pituitary.

In the present study, evidence supporting a central nervous system site of NPY action on ACTH secretion has been presented. Administration of pharmacological amounts of

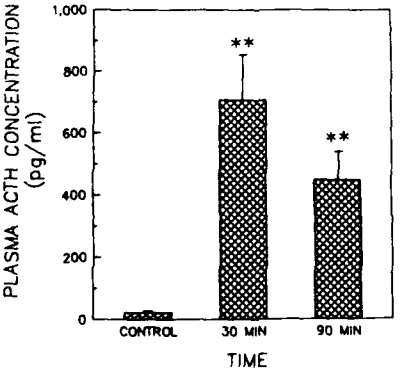

FIGURE 4. ACTH responses to insulin-induced hypoglycemia. Adult male rats were treated at 0900 hrs with 5 IU/kg recombinant human insulin (Humulin, Lilly), and were sacrificed 30 and 90 min after injection. The brains were rapidly removed from the skull, the ME was dissected from the fresh brain under microscopic visualization and peptide was extracted with boiling acetic acid. The remainder of the brain was frozen for later dissection. POA and MBH fragments were dissected from the frozen brain and were placed in boiling acetic acid. NPY concentrations were determined in these extracts by RIA. Blood was collected at the time of sacrifice in chilled plastic tubes containing EDTA. Plasma was separated by centrifugation and aliquoted for ACTH assay. Each point represents the mean plasma concentration of ACTH and the standard error of the mean in 8 animals. **-indicates significant differences at the 0.001 level vs the control (saline-treated) animals.

NPY to rats resulted in the activation of the hypothalamic-pituitary-adrenal axis.[19–21] This action could be mediated by alterations in noradrenergic tone. Härfstrand *et al.* have shown that NPY enhanced the turnover of NE in the PVN at high doses when ACTH levels were increased but at lower doses of NPY, ACTH levels and CA utilization decreased.[19] However, under in vitro conditions NPY reduced the release of NE into the medium.[40,41] It also has been shown that NPY modulates alpha-adrenergic receptor binding, so the metabolism of the amine may be linked to alterations in receptor function.[42] Therefore, the precise action of NPY requires further study.

Härfstrand *et al.* and Hisano *et al.* demonstrated that the NPY-containing neurons in the hypothalamic and brainstem regions possess glucocorticoid receptors, which could modulate the activity of NPY neurons.[43,44] Corder and colleagues demonstrated that pharmacological administration of dexamethasone increased the concentration of NPY in the basal hypothalamus.[45] However, whether this represents an increase in NPY synthesis or an inhibition of NPY release is presently unknown. Further questions regarding the interaction between the adrenal steroids and hypothalamic NPY neurons have arisen because Corder *et al.* were unable to demonstrate an effect of adrenalectomy on NPY concentrations in the PVN in spite of marked alterations in catecholamine metabolism.[46]

In the insulin-treated rats, complex changes in central NPY concentrations have been

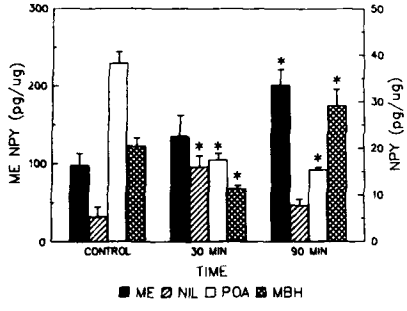

FIGURE 5. Hypoglycemia-induced changes in tissue NPY concentrations in several brain regions and the neurointermediate lobe of the pituitary. ME concentrations of NPY are compared to the *left axis* and are expressed as pg NPY per μg protein. Other areas should be compared to the *right axis* with the same units. See FIGURE 4 legend for further details. *-indicates statistical differences at the 0.05 level or better.

demonstrated. ACTH and corticosterone levels were rapidly increased in these animals. Concomitant with the change in ACTH is a marked depletion in POA and MBH NPY. These data suggest that NPY transport to the PVN (included in the POA) and release, therein, depletes NPY while activating CRH neurons. The interaction of NPY on CRH-containing neurons has previously been documented.[22] In addition, NPY interactions with vasopressinergic neurons also have been established[13,47,48] and according to the data of Plotsky *et al.* vasopressin plays an active role in insulin hypoglycemia-induced ACTH secretion,[35] although data from other laboratories suggest that CRH may also be an important mediator of the response to hypoglycemia.[36,37] Furthermore, an interesting study by Vallejo and Lightman[47] demonstrated that neonatal anti-NPY serum treatment resulted in aberrant vasopressin responses to dehydration in the adult rat. No apparent changes were observed in brain NPY stores; however, noradrenergic mechanisms appeared to be disrupted. This study highlights the importance of NPY in normal neuronal development and function in the hypothalamus. Therefore, the depletion of NPY in the anterior regions of the hypothalamus during stress could be related to the actions of this peptide on either CRH or vasopressin and suggests that NPY may transmit homeostatic information to critical hypothalamic areas.[18] Although Rivet *et al.*[46] were unable to alter hypothalamic NPY concentrations during a 30-min restraint paradigm, it is suggestive of a selective involvement of NPY in stress-induced ACTH secretion.

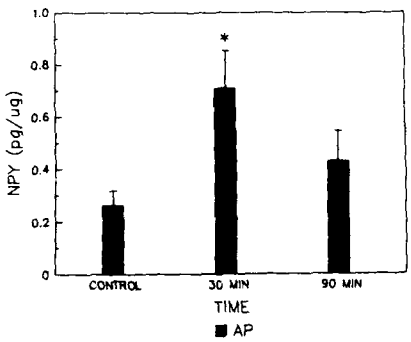

FIGURE 6. Hypoglycemia-induced changes in anterior pituitary NPY concentrations. See legends to FIGURES 4 and 5 for further details.

Increases in NPY concentrations in the neurointermediate lobe and median eminence are consistent with an increase in transport of NPY into nerve terminals in these areas during hypoglycemic stress, but whether release of this peptide is stimulated is currently unknown. The source of this peptide also is unknown although studies of Kerkerian *et al.* suggest that median eminence and neurointermediate lobe NPY may be derived from arcuate nucleus sources.[6]

Stress has been found to increase plasma concentrations of NPY in rats.[49,50] Hypoglycemia induced by insulin increased plasma concentrations of NPY in man as well.[51] Whether the pituitary gland or median eminence contribute to the rising systemic plasma concentrations of NPY is currently unknown but seems highly unlikely, because plasma concentrations of this peptide are very high (especially in rodents) and the tissue concentrations of the peptide are relatively low. If, however, the secretory rate was very high, then a contribution might be expected.

Endotoxin also altered anterior hypothalamic sources of NPY. However, the stress induced by treating rats with LPS was small as reflected by the small increase in ACTH concentrations. The time course of the response also is more prolonged than hypoglycemia-induced ACTH secretion, indicating that if earlier time points had been studied, a pattern of NPY changes more similar to hypoglycemia might have been observed.

Endotoxin-induced activation of the hypothalamo-pituitary-adrenal axis has recently been reported.[52] The increase in plasma concentrations of ACTH appear to be mediated by activation of hypothalamic CRH neurons.[52,53] However, endotoxin administration also modifies the activity of somatostatin-, thyrotropin-releasing hormone-, beta-endorphin- and vasopressin-containing neurons.[54–57] Activation of the immune response has also been reported to enhance the turnover of norepinephrine in the brain.[58] In the present study, it was found that NPY is depleted from POA stores 4 hrs after bacterial lipopolysacchride treatment but returns to control levels 24 hrs after injection. Significant changes in NPY concentrations in other brain regions or the pituitary were not observed. Since NPY is colocalized in brainstem noradrenergic neurons projecting to the PVN,[7,8] it would appear that NPY may be involved in transmitting homeostatic information to the hypothalamus. However, the significance of the NPY and noradrenergic colocalization in this response is currently unknown. It is possible that brainstem NPY fibers are not involved and the concomitant NPY changes are due to alterations in the responses of arcuate NPY-ergic neurons. Corder *et al.* also demonstrated that dexamethasone influences hypothalamic concentrations of NPY.[45] Therefore, the glucocorticoid increase associated with the administration of endotoxin may provoke the change in NPY. Further work in this area is required before adequate conclusions can be drawn.

NPY and its associated mRNA have been demonstrated in the cells of the immune system.[59] LPS administration is known to enhance the production of the cytokine, interleukin-1. Whether an activation of neuropeptide Y synthesis or secretion is associated with activation of the immune system is currently unknown. The possibility that peripherally produced interleukin-1 is responsible for the alteration in NPY also should be considered.

In conclusion, it is clear that NPY may act on neurons of the central nervous system to augment the activity of the hypothalamo-pituitary-adrenal axis. NPY-containing neurons in the brain are also influenced by the changes in the homeostatic environment of the animal and with norepinephrine may represent a pathway by which sensory inputs modulate neuroendocrine function. However, the current study also opens the possibility of a pituitary site of action for NPY and hence, peripheral sources of the peptide may play significant roles in changing pituitary hormone secretion.

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Neuropeptide Y and the Anterior Pituitary

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BACKGROUND

Neuropeptide Y (NPY) is a 36-amino-acid member of the pancreatic polypeptide family which was originally isolated by Tatemoto from porcine brain.[1] It shows a wide distribution throughout the mammalian nervous system, including the hypothalamus, and numerous endocrine systems and has been shown to affect the release of anterior pituitary hormones.[2]

Central administration of NPY in rat blocks the release of growth hormone (GH),[3] thyrotropin (TSH),[4,5] prolactin (PRL)[4,5] and, under some circumstances, luteinizing hormone secretion (LH).[3,5,6] NPY has also been reported to stimulate LH secretion in estrogen-primed female rats,[6,7] suggesting that this hormonal response to NPY depends critically on the animal's sex steroid background. When anterior pituitary cells from ovariectomised rats were tested in a perifusion system with various concentrations of NPY a significant dose-response stimulation of LH, GH and follicle stimulating hormone (FSH) release was observed.[3] Crowley noted a dose-dependent potentiation by NPY of LH stimulation by LH releasing hormone from anterior pituitary cell cultures obtained from ovariectomised rats.[8]

Recent recognition that the anterior pituitary is a source of bioactive compounds, including many neuropeptides that may have a local functional role, prompted this study. The aim of this investigation was to determine whether NPY is present in the anterior pituitary, and if so to investigate its cellular localisation and regulation by endocrine factors. As a peptide may be present in a tissue either as a result of local synthesis or from transport of exogenously produced peptide, we have assayed tissues for both peptide and messenger RNA (mRNA) content. The precise cellular location of NPY was determined by immunocytochemistry.

ANIMAL MODELS USED

Adult control and surgically manipulated Wistar rats were obtained from Interfauna Ltd. (Huntingdon, Cambridgeshire, UK) and experiments were timed to commence five days after surgery. They were caged in groups of five with a 13:11 h light-dark cycle

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(lights off at 1700 h) and an ambient temperature of 22°C. At the end of each experiment 9 animals from each group were alloted for radioimmunoassay and 6 for analysis of mRNA levels. For histochemical studies, 5 control and 5 thyroidectomised animals were examined. Control animals in all groups had sham surgery and daily injection of the vehicle as appropriate. All experiments were terminated after 28 days treatment except in the case of thyroid manipulation which ceased after 14 days. Estimation of relevant circulating hormone concentrations were in each case undertaken to confirm the efficacy of the endocrine treatments (data not shown).

The following treatment groups were studied.

1. *Thyroid manipulations.* Male animals were surgically thyroidectomised. A non-operated group received T_4 15 μg/100 g daily s.c.
2. *Adrenal manipulation.* Male rats were surgically adrenalectomised alone, or in combination with castration, and maintained on oral 0.9% NaCl. A non-operated group received dexamethasone 1 mg per kg daily s.c. Animals were sacrificed between 9.00 and 10.00 h daily.
3. *Estrogen manipulation.* Female rats were surgically ovariectomised and one half of the group received replacement 17 beta-estradiol 5 μg in 100 μl safflower seed oil daily s.c. A hyperestrogenised group received 17 beta-estradiol 2 mg s.c. on day 1 and day 21.

TABLE 1. Details of Conjugate, Antisera and Label Used in the RIA

Antigen	porcine NPY/BSA
Conjugation	glutaraldehyde
Antibody specificity	COOH-terminal
^{125}I-label	porcine NPY
RIA antibody dilution	1:120,000
Sensitivity (fmol/tube)[a]	0.6

[a]95% confidence limit from zero.

TECHNIQUES

Tissue Extraction and Analysis

Radioimmunoassay

The anterior pituitary lobe in each animal was separated from the neurointermediate lobe and extracted in 0.5 M acetic acid held at 100 °C for 10 min. Extracts were frozen and stored at −20°C until aliquots were assayed in duplicate as previously described.[9] Briefly, all assays were carried out in a total volume of 0.8 ml of 0.06 mol/litre phosphate buffer, pH 7.4, containing 10 mmol/litre EDTA, 45 μmol/litre BSA, and 20 kallikrein inhibiting units/ml aprotinin and were incubated at 4°C for 5 days. Antibody-bound label was separated from free label by adding to each tube 250 μl of a suspension containing 8 mg charcoal coated with clinical grade dextran (1:10 g charcoal). The tubes were centrifuged at 1600 X g for 20 min at 4°C, followed by immediate separation of the supernatant. Details of the assay are summarised in TABLE 1.

RNA Preparation and Northern Blot Analysis

Anterior pituitaries for RNA purification were pooled and snap frozen in liquid nitrogen and stored at −70°C prior to extraction using the acid guanidium thiocyanate-

phenol-chloroform method.[10] Total RNA was denatured and run on a MOPS 3-(N-morpholino)propane-sulphonic acid–formaldehyde–1% agarose–denaturing gel[11] before being transferred to Hybond-N membrane (Amersham, Amersham, UK). Following transfer, RNA was fixed by baking at 80°C for two hours before being probed with a radioactively labelled NPY cDNA probe. The rat specific complementary DNA (cDNA) probe was prepared by random hexanucleotide labelling[12] and denatured by boiling for five minutes before addition to the hybridisation solution. Filters were prehybridised at 42°C for two hours in a solution containing 50% formamide, 5 X sodium saline citrate (SSC),[13] 50 mmol sodium phosphate pH 6.8, 1 X Denhart's solution[13] and 100 μg/ml denatured sonicated salmon sperm DNA. Hybridisation was carried out at the same temperature for sixteen hours in the same solution to which had been added a ^{32}P labelled cDNA probe and dextran sulphate to a final concentration of 2 ng/ml and 10% (w/v) respectively. Following hybridisation, filters were washed twice at room temperature in a solution of 2 X SSC plus 0.2% sodium dodecyl sulphate (SDS) for five minutes before being washed twice at 60°C in a solution of 0.1 X SSC plus 0.2% SDS for thirty minutes. After washing, all filters were sealed into plastic bags and exposed to Kodak XAR-5 film with intensification screens at −70°C for one to ten days. Northern blots were analysed using a Joyce-Loebl Chromoscan 3 scanning densitometer (Joyce-Loebl, Gateshead, UK). The rat NPY clone was a kind gift from J. Dixon (Perdue University, Lafayette, IN).

Immunocytochemistry

Surgically thyroidectomised and control animals were perfused with 0.01 M phosphate buffered 0.15 M saline (PBS) pH 7.4, followed by Bouin's fluid. Pituitaries were removed and fixation continued for 1 hour before tissues were washed repeatedly in PBS containing 15% sucrose and 0.01% sodium azide to remove picric acid. After overnight washing the pituitaries were processed to paraffin wax. Paraffin sections (2 μm) of control and thyroidectomised rat pituitaries were immunostained using antiserum to synthetic porcine NPY, characterised previously[14] using the avidin biotin peroxidase method. Before immunostaining, endogenous peroxidase activity was blocked by incubation of sections in methanol containing 0.3% hydrogen peroxide for 30 min. For characterisation of the anterior pituitary cell type(s) immunoreactive for NPY, pairs of 2-μm serial paraffin sections were immunostained using anti-NPY (1:1000 dilution) and antisera to the classical anterior pituitary hormones (TSH, LH, GH, FSH, ACTH and PRL at dilutions of 1:4800, 1:3200, 1:3200 1:8000, 1:2000 and 1:800 respectively) obtained from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Bethesda, MD) as described previously.[15] Specificity of the hormone antisera was determined by using as a control antibodies to all rat pituitary hormones pre-incubated with 0.0001–1 nmol/ml of the respective antigens before being applied to the sections. Positively immunostained cells in each pair were compared by light microscope to identify coexistence, and the results were confirmed by comparison of high power photomicrographs.

Statistical Analysis

All results for RIA are presented as mean ± SEM. Comparison between groups was initially assessed by one-way analysis of variance and, when indicated, by a non-paired Student *t* test.

TABLE 2. Densitometry Results of Anterior Pituitary Hormone and NPY mRNA Analysed by Northern Blots following Endocrine Manipulation[a]

	Classical Hormone mRNA	NPY mRNA
C	100%	100%
T_4	bTSH undetectable	128
Tx	bTSH >725	21,760
Ax	POMC 260	88
AC	POMC 220	118
Dx	POMC 21	121
Ox	bLH >1000	230
OR	bLH 14	104
E_2	bLH 2	112

[a]Control values were arbitrarily assigned a value of 100 optical density units and all other groups a percentage of this. C = control, T_4 = hyperthyroid, Tx = thyroidectomised, Ax = adrenalectomised, AC = combined Ax + castration, Dx = dexamethasone treated, Ox = ovariectomised, OR = Ox + estrogen replacement, E_2 = hyper-estrogenised.

OBSERVED CHANGES IN NPY

Anterior Pituitary Hormone mRNA Expression

There were marked differences in the expression of anterior pituitary target cell hormone mRNA following each endocrine manipulation, as shown in TABLE 2. Each hormone mRNA showed evidence of classical negative feedback regulation as expected and thus confirmed the efficacy of the manipulation.

Anterior Pituitary NPY Peptide and NPY mRNA Expression

The results of endocrine manipulation on NPY mRNA and peptide content are presented in TABLES 2 and 3 respectively. In the male rat the most interesting effects were observed following thyroidectomy when a dramatic increase in NPY content was noted (335 ± 58 fmol/gland vs control, 15 ± 4, p <0.001). This was accompanied by a

TABLE 3. Anterior Pituitary NPY Content following Endocrine Manipulation[a]

Group	NPY-IR
C	15 ± 4
T_4	12 ± 3
Tx	**335 ± 58
C	17 ± 3
Ax	16 ± 3
AC	**7 ± 3
Dx	18 ± 3
C	20 ± 2
Ox	*35 ± 4
OR	21 ± 3
E_2	16 ± 2

[a]Values are fmol/gland (mean ± SEM, n = 9). Abbreviations as in TABLE 2.
*versus control, p <0.01.
**versus control, p <0.001.

>200-fold increase in NPY mRNA expression relative to control pituitaries (FIG. 1). Chronic high-dose T_4 treatment failed to affect either NPY content or mRNA expression. Similarly, pituitary NPY content was unchanged in the adrenalectomised and dexamethasone treated groups. However, NPY content was decreased following combined adrenalectomy and castration (7 ± 3 vs 17 ± 3 fmol/gland, $p < 0.001$). This was accompanied by a 20% increase in NPY mRNA levels. In the female rat, ovariectomy resulted in a reversible doubling in both NPY peptide and mRNA expression. However, estrogen treatment in pharmacological doses failed to alter pituitary NPY expression.

Immunocytochemistry

The results of immunocytochemistry are shown in FIGURE 2. Large, polygonal cells were sparsely distributed in control rat anterior pituitaries. There was no NPY-immunoreactivity visible in the neurointermediate lobe. Following thyroidectomy the staining intensity, size and frequency of NPY-positive cells increased, and they retained their

FIGURE 1. Northern blot analysis of NPY mRNA from anterior pituitaries of control and thyroid manipulated animals. All lanes contained contained 40 μg of RNA except for the lane marked 5, which contained 5 μg of total RNA. Abbreviations as in TABLE 2. *Upper and lower dashes* on the left indicate the positions of 28S and 18S ribosomal RNAs, respectively.

polygonal shape. In both control and thyroidectomised rats these NPY-immunoreactive cells were consistent in appearance with thyrotropes. Colocalisation studies revealed that a proportion of TSH-immunoreactive cells in both control and hypothyroid pituitaries were positive for NPY. This suggests that the NPY immunoreactivity is found in a subset of thyrotropes. No other anterior pituitary cell types were positive for NPY immunoreactivity.

CONCLUSIONS

These results demonstrate the presence of NPY and its prohormone encoding mRNA in the adult rat anterior pituitary and their coordinate regulation by endocrine status, particularly thyroid hormone levels. These findings suggest that NPY regulation in the pituitary occurs predominantly at the gene transcriptional level. In addition, NPY immunoreactivity has been localised to a population of thyrotropes in both the normal and hypothyroid pituitary.

The physiologic role of NPY synthesised within the anterior pituitary is unknown. Most studies which have looked at the direct effects of NPY on anterior pituitary hormone secretion have concentrated primarily on gonadotrope function and have produced inconclusive results.[2] NPY stimulated in a dose-responsive manner the release of LH, GH and FSH from anterior pituitary cells harvested from ovariectomised rats in a perifusion column[3] while the addition of NPY to 4 day monolayer cultures of anterior pituitary cells from intact female rats produced no significant effect on the release of any of the pituitary hormones tested.[7] The addition of NPY to hemipituitary fragments from intact male rats had no effect on the release of LH, FSH or TSH.[16] However, the addition of NPY in combination with LH releasing hormone to cultures of either hemipituitary fragments or dispersed cultured pituitary cells from ovariectomised rats has been shown to significantly

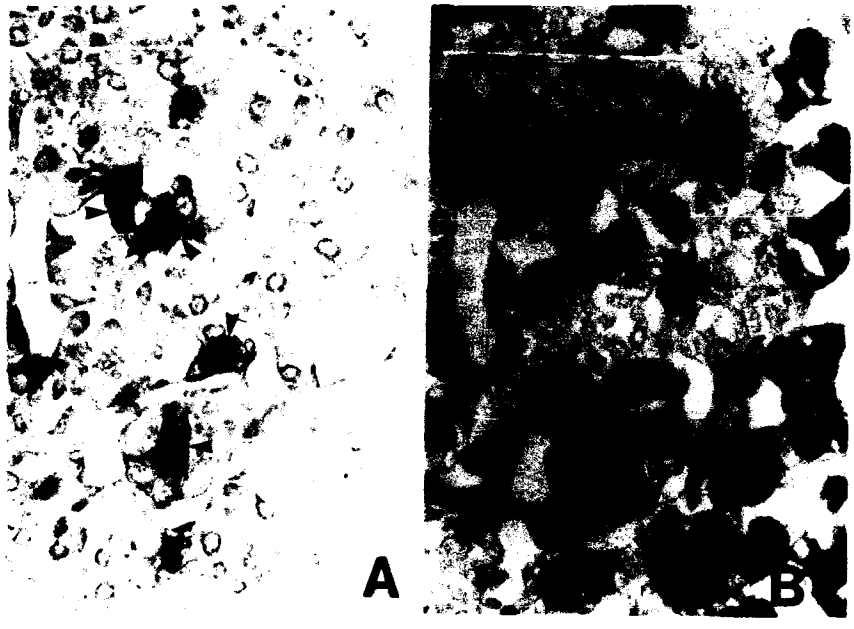

FIGURE 2. Serial 2 μm sections of anterior pituitary of a thyroidectomised rat immunostained for **(A)** NPY and **(B)** TSH, respectively. NPY is colocalised with TSH in a subset of thyrotropes as indicated by the *arrowheads*. Original magnification X450.

enhance the LH releasing effect of LH releasing hormone.[8] Interestingly, passive immunoneutralisation with NPY antiserum has been shown to inhibit the LH surge in ovariectomised rats presumably by a pituitary action.[17] It is likely that NPY effects on gonadotrope function are significantly influenced by the ambient sex-hormonal environment.

The localisation of NPY immunoreactivity to the thyrotrope and the dramatic effects of hypothyroidism on NPY expression were unexpected observations. It is possible that changes in pituitary cell populations[18] could contribute to the changes in NPY expression but it is doubtful that such dramatic changes could result solely from alterations in cell numbers. A more likely explanation is that NPY is processed in parallel with TSH. As there are no reports of NPY regulating thyrotrope function or secretion it is possible that NPY is released from the thyrotrope to influence neighbouring cells in a paracrine fashion. The

doubling of anterior pituitary NPY following ovariectomy coupled with its unresponsiveness to high-dose estrogen treatment and male castration (data not shown) may further reflect the intrinsic differences in the gonadotrope response to NPY which appears to be dependent on the sex-steroid status. Similarly, the result obtained in the combined adrenalectomised and castrated group might reflect a similar divergence in function.

These results strongly suggest that NPY is synthesised within the rat anterior pituitary and that this synthesis is dramatically increased in the hypothyroid state. The application of in-situ hybridisation techniques and receptor mapping studies should further enhance our understanding of the intrapituitary role that NPY plays.

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POSTER PAPERS

Conformational Analysis of the Neuropeptide Y Fragment NPY(18–36) by ^{1}H-NMR and Computer Simulation[a]

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INTRODUCTION

In the ongoing effort to elucidate the structural requirements of NPY necessary for both receptor selectivity and efficacy, the conformational preferences of the Y_2-receptor selective ligand NPY(18–36) have been investigated using 500 MHz ^{1}H-NMR and computer simulations. Towards the development of conformationally constrained receptor specific agonists and antagonists, elucidation of the conformational preferences of NPY(18–36) and related analogs is essential. We report here the results of the investigation of NPY(18–36).

METHODS

NPY(18–36) was synthesized by solid phase methodology and purified by RPHPLC.[1] The conformational characterization was carried out using ^{1}H-NMR and NOE-restrained energy minimizations as well as high and low temperature dynamics simulations. The proton resonances were assigned using 2D COSY, HOHAHA and ROESY techniques.[2–4] All NOE experiments used for determination of the preferred conformations of NPY(18–36) were carried out in dimethylformamide-d_7 (DMF-d_7).

Computer simulations were carried out on a SGI Iris 4-D using the CHARMm and QUANTA simulation programs. Fifty random conformations (random ϕ, ψ and χ_1 angles), a fully extended conformation and a right-handed α-helical conformation were

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subjected to minimization with five successive additions of long-range NOEs. All conformers were then allowed to relax (*i.e.*, no constraints). The initial structure resulting in the conformer with the best overall agreement to NOE and $J_{NH-\alpha}$ (the α-helix) was then subjected to constrained high temperature (750 K) dynamics punctuated by unconstrained minimizations.

RESULTS AND DISCUSSION

Previous work involving CD spectroscopy revealed that NPY(18–36) existed as a random coil in water. However, upon addition of trifluoroethanol a high degree of

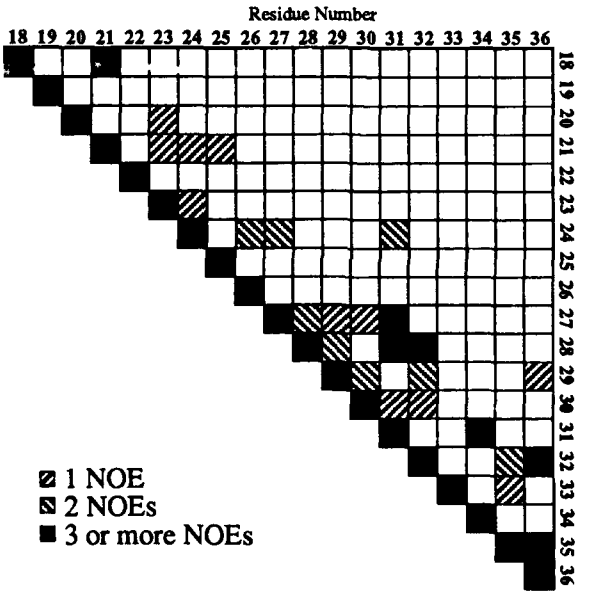

FIGURE 1. NOE information for NPY(18–36) from ^{1}H-NMR in DMF-d_7. Residue number refers to position in NPY(1–36). Primary sequence of NPY(18–36): Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2.

α-helicity was observed.[1] Preliminary NMR in D_2O and DMSO-d_6 showed a high degree of resonance degeneracy and a lack of NOEs, likewise suggesting a random configuration. DMF-d_7 was chosen as the solvent of choice based upon its known structure stabilizing properties. Indeed, ample NOEs and resonance dispersion have been observed for over a dozen other NPY(18–36) analogs, some as short as ten amino acids (unpublished results).

Of the over 150 NOEs identified for NPY(18–36), 48 were classified as long range (*i.e.*, interresidue, excluding backbone i to i + 1 NOEs, FIG. 1). The initial minimization procedure applied to 52 starting conformations (one extended, one α-helix, fifty random) revealed the α-helix to be superior to all others as a starting structure, despite the fact that it lost much of its helical character over the course of the minimizations. Unconstrained

minimizations of 100 structures resulting from 10 ps of constrained high temperature dynamics of the "α-helix" revealed six major families of conformers. The lowest energy members of each of the six families all showed good agreement with the NOE results and $J_{NH-\alpha}$ coupling constants (FIG. 2).[5]

For all six low energy structures certain overall conformational properties remained conserved. Of particular interest was the hydrophobic core present in all, made up of the residues Ala,[23] Leu,[24] Ile,[28] Leu[30] and Ile.[31] In the case of NPY(18–36) (as opposed to residues 18 to 36 within NPY(1–36)) the formation of a hydrophobic core was reasonable, in that it most efficiently shielded the nonpolar side chains of Ala, Leu and Ile.

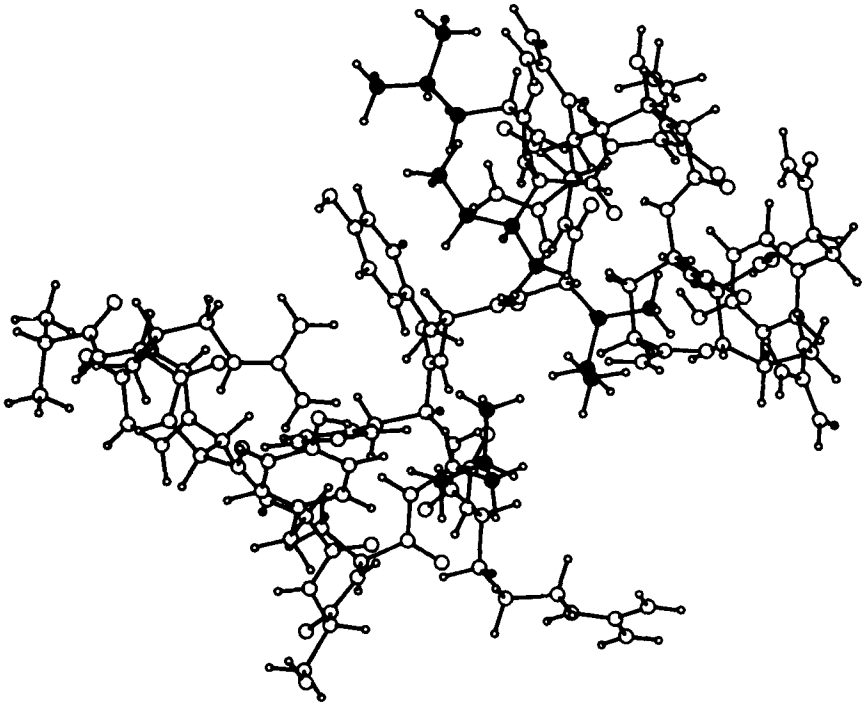

FIGURE 2. Minimum energy conformer of NPY(18–36) derived from constrained high temperature dynamics. N-terminus is at *extreme left*, C-terminus is at *upper right*. Hydrophobic cleft denoted by *filled* atom centers of side chains from Ile and Leu residues (*middle top* to *middle bottom*).

The detailed conformational analysis of the many NPY(18–36) analogs is currently under way. It is anticipated that important structure-activity relationships will become clear.

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Characterization of Neuropeptide Y Immunoreactivity in Dog Plasma by High Performance Liquid Chromatography and Radioimmunoassay

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INTRODUCTION

Fragments of neuropeptide Y (NPY) with varying degrees of physiological and immunological activity may circulate in the blood. NPY in plasma extracts has been routinely quantified by radioimmunoassay (RIA) using polyclonal antibodies. While these determinations are sensitive and provide useful information, the levels of NPY may represent a mixture of NPY and peptide fragments of varying biological and immunological potency. The aim of this study was to develop a method for separation and quantitation of NPY immunoreactivity (NPY-ir) in dog plasma as a first step towards understanding the processing of the peptide and characterizing the circulating levels of the peptide and its fragments.

MATERIAL AND METHODS

Venous blood (10 ml) was collected into ice chilled tubes containing heparin (20 IU/ml blood) and aprotinin (500 KIU/ml blood) from 20 normal conscious male mongrel dogs weighing between 18 and 25 kg. The effects of either a short or a long-lasting barbiturate on venous levels of NPY were evaluated by a comparison of the effects of anesthesia induced by either sodium pentobarbital (20 mg/kg IV) ($n = 5$) or sodium thiamyal (surital, 20 mg/kg IV) ($n = 6$). Samples simultaneously collected from a vein and an indwelling arterial catheter of a conscious dog were used for high performance liquid chromatography (HPLC) analysis. Blood samples were centrifuged at 2100 x g for 20 minutes at 4°C and the plasma separated and stored at −20°C. Plasma samples were thawed at 4°C. NPY was extracted from plasma proteins using a Waters octadecasilane reverse phase cartridge, which was activated by sequentially washing with 5 ml of meth-

anol, tetrahydrofuran, hexane, methanol and distilled water. Plasma was applied to the Sep-Pak, washed with distilled water (10 ml) and eluted with 60% acetonitrile. Fractions were evaporated to dryness and either quantified by RIA or stored at −20°C for HPLC followed by RIA. ^{125}I-NPY (1000 cpm) and synthetic porcine NPY (range 100–1000 pg/ml) were added to plasma samples before extraction to determine radiolabel and immunoreactive recoveries. The RIA was carried out according to the method of Allen *et al.* and the characteristics of the assay are summarized in TABLE 1.

NPY peptides and fragments were separated on a LKB 2150 gradient system equipped with a Vydac C_{18} column (4.6 x 150 mm, Hesperia, CA) and a 3.2 x 15 mm Newguard C_4 guard column (Brown Lee Labs, Santa Clara, CA). The column was equilibrated with 60% mobile phase A [0.13% heptafluorobutyric acid (HFBA)] and 40% mobile phase B

TABLE 1. Neuropeptide Y Radioimmunoassay Characteristics Antiserum-Specificity

Peptide	% Cross-Reactivity
Porcine neuropeptide Y(1–36)	100%
Porcine neuropeptide Y(16–36)	33%
Human neuropeptide Y(1–36)	100%
Human neuropeptide Y(20–36)	66%
Human neuropeptide Y(22–36)	100%
Human neuropeptide Y(26–36)	100%
Human pancreatic polypeptide	0.02%
Avian pancreatic polypeptide	0.007%
Peptide YY	0.003%
Vasoactive intestinal peptide	0.001%
Rat corticotropin releasing factor	0.001%
Human growth hormone releasing factor	0.001%
Glucagon	0%
Secretin	0%
Sensitivity	
ED(20) = 225 pg/tube	
ED(50) = 116 pg/tube	
ED(80) = 58 pg/tube	
Reproducibility	
Intraassay variation = 10%	
Interassay variation = 14%	
Recovery	
Synthetic peptide = 76 ± 8%	
Radiolabeled peptide = 80 ± 5%	

(80% acetonitrile/0.13% HFBA) at a flow rate of 1 ml/min. Plasma extracts were dissolved in 40% mobile phase B and applied to the column. NPY and peptide fragments were eluted with a 24 min gradient of 40–47% mobile phase B. Fractions (0.2 ml) were collected at 12-sec intervals and evaporated to dryness. The dried fractions were reconstituted in the phosphate buffer (0.06 M, pH 7.4) and quantified by RIA.

RESULTS AND SUMMARY

In conscious dogs, plasma levels of NPY-ir in venous blood averaged 126 ± 11 pg/ml (n = 20). The circulating levels of NPY were not influenced by anesthesia induced by

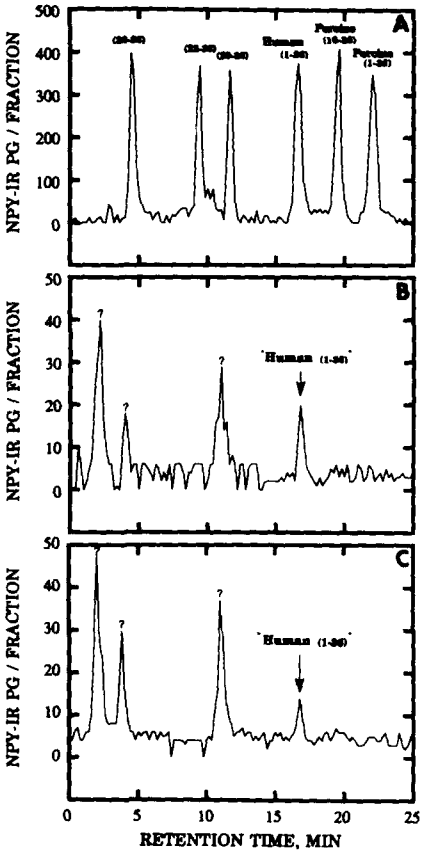

FIGURE 1. High pressure liquid chromatographic separation of NPY and its immunoreactive fragments. In panel **(A)** the retention times for standards [NPY(26–36), NPY(22–36), NPY(20–36), human NPY(1–36), porcine NPY(16–36) and porcine NPY(1–36)] are shown. HPLC analysis of normal conscious arterial (panel **B**) and venous (panel **C**) plasma shows a fragment with retention times similar to human NPY and a number of unknown peaks.

either sodium pentobarbital (92 ± 17 vs 138 ± 42 pg/ml) or sodium thiamyal (109 ± 23 vs 113 ± 22 pg/ml). Canine plasma contained a NPY fragment with properties similar to the human, but not porcine form FIG. 1.) This fragment, probably canine NPY(1–36), was found in both arterial and venous samples. It accounted for approximately 15% of the total NPY-ir, and thus is not a major circulating component in dog plasma. Three other NPY fragments amounting to 42, 14, and 32% of the total NPY-ir had retention times that differed from known standards. In summary, this is the first demonstration of NPY and its carboxy-terminal immunoreactive components in plasma.

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Peptide YY/Neuropeptide Y Receptors in Small Intestine

Characterization, Signal Transduction, and Expression during Cell Differentiation

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Peptide YY (PYY) and neuropeptide Y (NPY) are present in high concentrations in the intestines. PYY is localized in endocrine cells of the mucosa (colon, rectum, ileum) whereas NPY is strictly a neuropeptide. NPY-containing nerve fibers form a dense network in the lamina propria which underlies the small intestinal epithelium (see REF. 1 for review). In 1986, we reported the properties of a new receptor for PYY and NPY in rat intestinal epithelium.[2] This receptor has been characterized by using ^{125}I-labeled PYY as a tracer and is PYY-preferring since it binds PYY (Kd = 0.1 nM) with a 5-times-higher affinity than NPY.[2] Concomitantly, PYY and NPY were shown to be potent inhibitors of fluid and electrolytes secretion in small intestine (see REF. 1 for review), providing a functional significance for intestinal epithelial PYY-NPY receptors.[2] We have further shown recently that PYY and NPY inhibit VIP- or prostaglandins-stimulated cAMP production in rat small intestinal epithelial cells.[3] The receptor mediating this inhibition[3] is also PYY-preferring as assessed pharmacologically with PYY, NPY and several fragments of the peptides[3] including PYY 22–36 which appears to be a potent agonist.[3]

In small intestinal epithelium, cells originate in the crypts and migrate onto the villi (FIG. 1). Crypt cells proliferate rapidly, but as they migrate they stop to divide and they differentiate into a major population of columnar absorptive cells. The villus is the site of ions and nutriments absorption whereas the crypt is believed to be the site of Cl^- secretion. We have recently determined the distribution of PYY-NPY receptors along the villus-crypt axis (FIG. 1). By using monoiodinated PYY (from NEN) as a tracer, we have shown that the binding capacity of receptors decreases markedly from crypt to villus (REF. 4 and FIG. 1). Scatchard plots are linear in both cell populations (FIG. 1): B_{MAX} are 166 and 21 fmol/mg protein and Kd are 0.1 and 0.05 nM in membranes prepared from crypt and villus cells, respectively (REF. 4 and FIG. 1). The receptor is PYY-preferring in both compartments.[4] PYY and NPY inhibit VIP-stimulated cAMP production in crypt cells but not in villus cells (REF. 4 and FIG. 1). Therefore, PYY-preferring receptors, negatively coupled to cAMP production system, are preferentially expressed in crypt cells where they mediate the potent anti-secretory actions of PYY and NPY on water and electrolytes (see REF 1 for review).

As shown in nervous tissues, NPY/PYY binding sites occur in two subtypes, Y1 and Y2, which differ in specificity but accept NPY and PYY equally well. The Y1 receptor does not bind NPY 13-36 whereas the Y2 receptor binds NPY 13-36 with a good affinity.[5] The PYY-preferring receptor characterized in small intestine[1-4] resembles the Y2 receptor in that it binds PYY 22-36 with a good affinity[3] but differs from it since it discriminates between PYY and NPY.[1-4] In order to further explore this issue, monoiodinated radioligands of PYY and NPY have been prepared by reverse phase HPLC as described[6] with

modifications (FIG. 2). Since PYY 22-36 binds rather well to intestinal PYY receptors,[3] we have used [^{125}I-Tyr^{1}] monoiodo PYY to further characterize PYY receptors in rat small intestine (FIG. 2). The receptor labeled by this radioligand discriminates between PYY, NPY, and pancreatic polypeptide as did radiolabeled PYY tracers used previously.[1-4] This further confirms the expression of a PYY-preferring receptor in rat

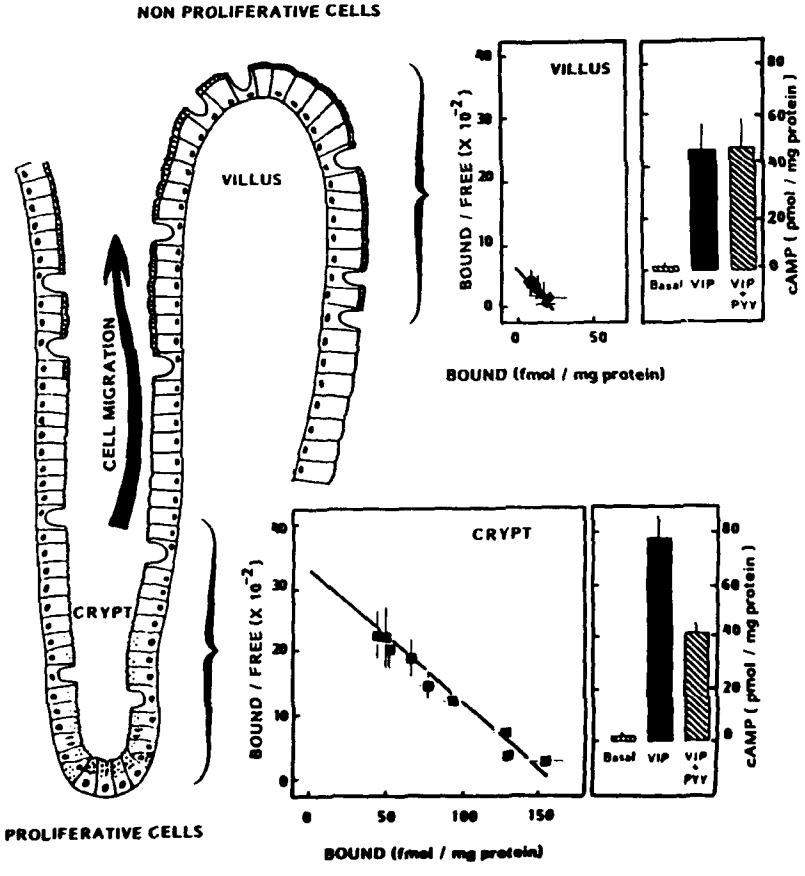

FIGURE 1. Expression of PYY-preferring receptors during epithelial cell differentiation along the rat small intestine villus-crypt axis. Proliferative crypt cells were separated from mature villus cells as described.[4] Monoiodinated PYY (NEN) was used to characterize receptors in crude membranes from crypt cells (■) and villus cells (●). cAMP was measured in isolated cells after incubation with 1 μM VIP alone or in the presence of 1 μM PYY. See REF. 4 for other details.

small intestine. [^{125}I-Tyr^{36}] monoiodo NPY, which was used to characterize Y1 and Y2 receptors in nervous tissues,[5,6] was also tested in intestinal cells (FIG. 2). This tracer gives a high amount of specific binding but the pattern of competitive inhibition by PYY, NPY, and PP is quite different from that observed with [^{125}I-Tyr^{1}] monoiodo PYY. Indeed, [^{125}I-Tyr^{36}] NPY binding is inhibited 1) by NPY with a high IC_{50} (160 nM), and 2) by

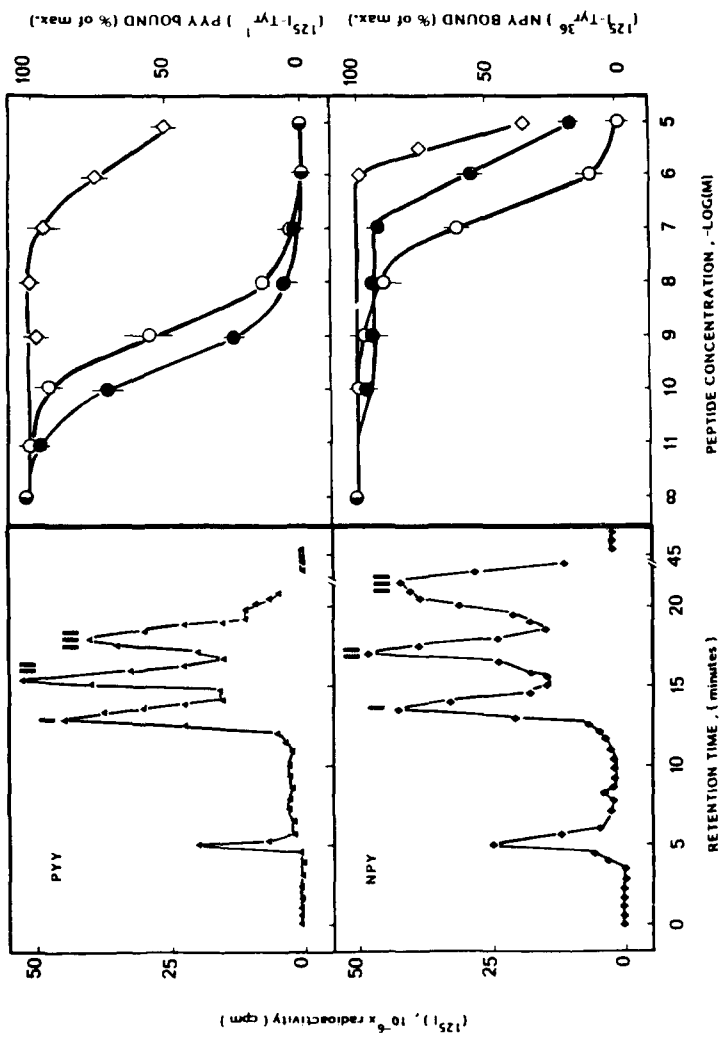

FIGURE 2. Purification of radiolabeled PYY and NPY by reverse phase HPLC and binding of [^{125}I-Tyr^{1}] PYY or [^{125}I-Tyr^{36}] NPY to membranes from rat small intestinal epithelial cells. *Left*. Representative profiles of radioactivity from a purification of radiolabeled PYY (*top*) or NPY (*bottom*) on a Nucleosil C18-300 column eluted isocratically with 31% acetonitrile. The peak eluted at 5 min is unreacted free ^{125}I. Peaks I, II and III were shown to be [^{125}I-Tyr^{1}] monoiodo-, [^{125}I-Tyr^{36}] monoiodo-, and [^{125}I-Tyr^{1}, ^{125}I-Tyr^{36}] diiodo- PYY or NPY, respectively.[6] *Right*. Inhibition of [^{125}I-Tyr^{1}] monoiodo PYY (*top*) or [^{125}I-Tyr^{36}] monoiodo NPY (*bottom*) binding by unlabeled PYY (●), NPY (○) or rat PP (◇) in membranes prepared from rat jejunal epithelial cells. Membranes (200 μg protein/ml) were incubated with 0.05 nM tracer for 2 h at 15°C. See REFS. 2–4 for other details.

unlabeled peptides with the following order of potency NPY>PYY>PP (FIG. 2). These data suggest that a low affinity NPY-preferring binding site may coexist with high affinity PYY-preferring receptors in rat small intestinal epithelium. The biological significance, if any, of the low affinity NPY-preferring binding site and its distribution along the crypt-villus axis remains to be determined.

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Structural Characterization of Y_1 and Y_2 Receptors for Neuropeptide Y and Peptide YY

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Two receptor subtypes for peptide YY (PYY) and neuropeptide Y (NPY) named Y_1 and Y_2 have been proposed to exist based on pharmacological studies.[1,2] In order to identify and structurally characterize Y_1 and Y_2 receptors we covalently cross-linked [^{125}I-Tyr^{36}]PYY to its receptors. Affinity labeling of the Y_1-receptor protein was analyzed in membranes from the MC-IXC human neuroblastoma cell line. Analysis by SDS-polyacrylamide gel electrophoresis and autoradiography resulted in labeling of a major band centered at M_r = 70,000, and a smaller band at 45,000. The labeled bands were unaffected by reducing agents (FIG. 1). The labeling of the M_r = 45,000 band was greater in the absence of protease inhibitors, suggesting that it is a degradation product of the larger band. For characterization of Y_2 receptors we utilized two different tissues; rat hippocampal membranes and rabbit kidney tubule membranes. In both tissues affinity labeling of PYY binding proteins resulted in labeling of a major protein centered at M_r = 50,000, which was unaffected by reducing agents (FIG. 2). For both the Y_1 and Y_2 receptors the binding to the receptors and the intensity of the labeled bands were inhibited in a parallel dose-dependent manner by increasing concentrations of unlabeled PYY.[3] Efficient labeling of Y_2-receptor proteins was obtained using a number of different homo- and heterobifunctional cross-linking reagents, whereas labeling of Y_1-receptor proteins was obtained only when N-5-azido-2-nitrobenzoyloxysuccinimide (ANBNOS) was employed for cross-linking followed by exposure to UV light. To determine whether the receptors were glycoproteins the detergent solubilized receptor-hormone complexes were exposed to different agarose-coupled lectins. The cross-linked Y_1 and Y_2 receptors were almost completely absorbed by wheat germ agglutinin agarose. The Y_1 receptor was partially absorbed by ricin communis II, and the Y_2 showed partial absorption to concanavalin A. These absorptions were in all cases blocked by the appropriate hapten sugar. These results indicate that the Y_1 and Y_2 subtypes of NPY and PYY receptors, previously characterized pharmacologically, are structurally distinct glycoproteins, not disulfide-linked to other subunits.

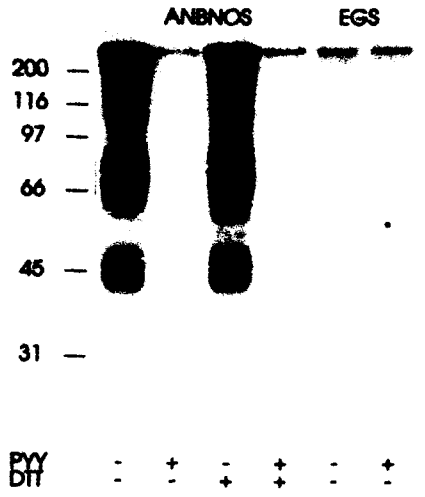

FIGURE 1. Affinity labeling of the Y_1 receptor in a human neuroblastoma cell line, MC-IXC, with radiolabeled PYY. Membranes (200 μg) were incubated with 250 pM radiolabeled PYY, washed and cross-linked with 0.1 mM ANBNOS or 0.5 mM EGS. Reducing agent (50 mM DTT) and unlabeled PYY (1 μM) were added as indicated. (From Sheikh and Williams.[3] Reprinted by permission from the *Journal of Biological Chemistry*.)

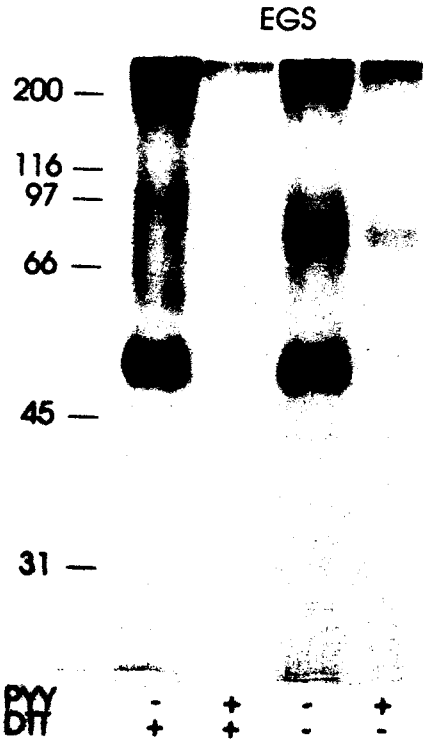

FIGURE 2. Affinity labeling of the Y_2 receptor in rat hippocampus with radiolabeled PYY. Membranes (200 μg/ml) were incubated with 200 pM radiolabeled PYY, washed free of unbound ligand, and cross-linked with 0.5 mM EGS. Unlabeled PYY (1 μM) and dithiothreitol (50 mM) were added as indicated. (From Sheikh and Williams. Reprinted by permission from the *Journal of Biological Chemistry*.)

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Peptide YY Receptors in Mammalian and Avian Brains

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The ligand binding technique developed during the past two decades has identified a great multiplicity of neurotransmitter receptors, including those for NPY and PYY.[1-11] While most of the CNS immunoreactivity for this PP family of peptides is NPY, PYY-containing neurons are also present in the brain. This provides an anatomical basis for the effects of NPY and PYY on brain functions. In the present study, we summarize the PYY receptors in mammalian and avian brains.

Direct binding of radioactive peptides to their target cells identifies receptors by their affinity and specificity. Two classes of [^{125}I]PYY binding sites were observed in porcine brain membrane preparations.[6,7] The calculated binding affinity of 0.139 nM for the high affinity component is similar to the value reported for PYY binding to membranes prepared from rat intestinal epithelium (kd = 0.4 nM), which is consistent with the venous blood level of PYY observed after a meal. Brain PYY receptors also exhibit high specificity. However, PYY and NPY receptors do not distinguish between PYY and NPY (FIG. 1), suggesting that PYY and NPY regulate brain function through interaction at common receptor sites, a notion also suggested from biological studies. PYY receptor binding requires not only the C-terminal portion of the molecule but also the N-terminal portion, and is linked to a second messenger system including adenylate cyclase inhibition via G proteins.

TABLE 1 shows the distribution of PYY receptors in the brain, which are abundant in the hippocampus, amygdala, hypothalamus, and the pituitary gland. In dogs and rodents high binding activity was also observed in the thalamus and cerebral cortex, suggesting species differences. Autoradiographic studies with radiolabeled NPY and PYY demonstrated that PYY and NPY receptors are quite similarly distributed throughout the brain, although somewhat differential localizations were observed, *e.g.*, in the mamillary body, thalamus, or the cerebellum.[10-12]

The PYY receptors in brain and intestinal epithelial membranes were further characterized after covalent labeling with chloramine T-iodinated PYY using homobifunctional cross-linker disuccinimidyl suberate.[5,7] Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography demonstrated that PYY receptors are protein of mol wt 50 kDa with no disulfide-linked subunits (the same as that of NPY receptors), and are found in various regions of the brain and intestinal epithelium across the species. In the chicken brain, however, PYY receptors are found as a 67-kDa protein band which does not discriminate either PYY, NPY or avian PP well.[8] The APP and NPY receptors are also the same 67-kDa proteins, with PYY, NPY and APP acting at either of these receptors. These results indicate that chicken brain also has receptors specific for

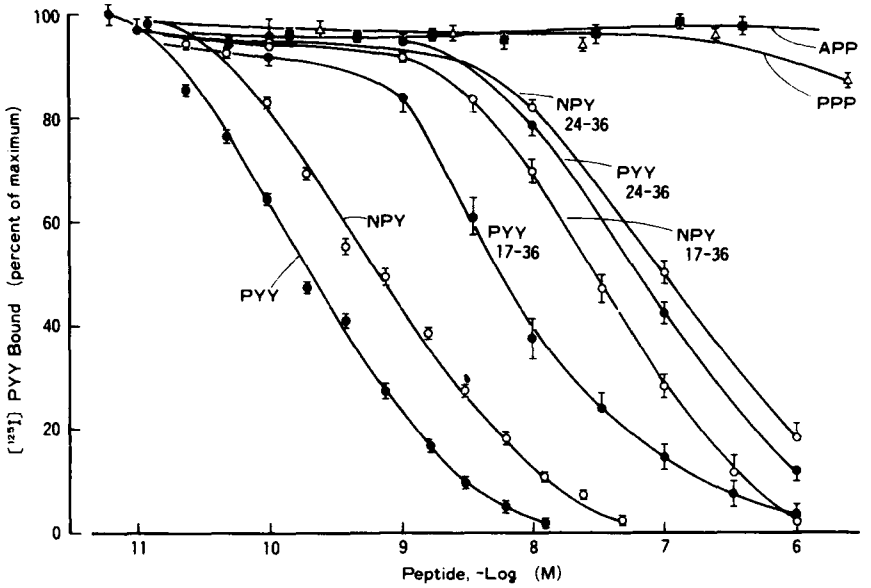

FIGURE 1. Inhibition of [^{125}I]PYY binding to porcine hippocampal membranes by PYY analogs. Membranes were incubated with 40 pM [^{125}I]PYY for 5 h at 25°C. Varying concentrations of analogs were added as shown. Nonspecific binding was subtracted from total binding and specific binding was expressed as a percentage of maximal specific binding.

PYY and NPY, and that PYY, NPY, and APP act in the brain through interaction at multiple receptor sites which are quite similar to, and shared by, other members of the PP family.

We have recently found that porcine brain has binding sites specific for APP (TABLE 1). The APP binding sites are more specific for APP than those in avian brain, since

TABLE 1. Regional Distribution of PYY, NPY, and APP Receptors in the Brain

	Specific PYY Binding (fmol/mg Protein)			Specific NPY Binding (fmol/mg Protein)	Specific APP Binding (fmol/mg Protein)
Brain Area	Pig	Dog	Guinea Pig	Pig	Pig
Cerebral cortex	2.5 ± 0.9	5.6 ± 1.4	11.5 ± 2.8	0.11 ± 0.05	9.9 ± 1.9
Olfactory bulb	4.2 ± 1.2	3.0 ± 0.8	4.8 ± 1.8	0.41 ± 0.20	8.5 ± 1.7
Caudate nucleus	17.7 ± 4.9	15.1 ± 2.2	12.4 ± 3.1	0.43 ± 0.15	9.4 ± 2.1
Hippocampus	84.5 ± 13.6	22.6 ± 7.1	14.6 ± 3.5	3.05 ± 0.80	11.2 ± 1.7
Amygdala	28.6 ± 5.8	12.4 ± 2.0	14.0 ± 4.5	0.64 ± 0.13	10.0 ± 1.7
Thalamus	4.0 ± 0.9	18.3 ± 3.2	6.9 ± 1.3	0.18 ± 0.10	9.5 ± 1.6
Hypothalamus	34.3 ± 9.1	13.2 ± 3.4	10.1 ± 2.8	0.88 ± 0.14	9.7 ± 1.8
Pituitary gland	58.9 ± 13.6	12.4 ± 3.6	10.6 ± 0.7	2.85 ± 0.76	16.9 ± 2.7
Midbrain	11.3 ± 2.5	12.2 ± 2.6	11.9 ± 2.9	0.26 ± 0.13	9.1 ± 1.1
Pons	9.4 ± 2.4	5.6 ± 2.0	8.1 ± 1.8	0.40 ± 0.10	9.4 ± 1.4
Medulla oblongata	16.3 ± 3.0	7.9 ± 0.8	6.9 ± 1.5	1.11 ± 0.27	7.5 ± 1.8
Cerebellum	3.0 ± 1.5	0.4 ± 0.2	8.3 ± 2.2	0.31 ± 0.10	9.6 ± 1.0

porcine tissue APP binding cannot be inhibited by NPY, PYY or PPP, whereas in chicken tissue all peptides of this family inhibit APP binding. Although the relatively low affinity of the porcine APP binding site does not allow consideration of its functional significance, it is possible that some endogenous peptide related to APP may exist and serve a specific function in porcine brain.

The identification of all of the PP family peptides and their receptors, especially their structures form a basis for the rational design of new, potent and selective drugs for each receptor subtype, an important advancement in neuroscience.

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Neuropeptide Y Inhibits Phosphorylation of 87-kDa Protein in Rat Vas Deferens[a]

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INTRODUCTION

A major substrate for protein kinase C phosphorylation, the 87-kDa protein, has been identified in brain, spleen, lung and vas deferens of several species.[1] In vas deferens, NPY-containing sympathetic nerves are abundant in the smooth muscle wall.[2] We have investigated the effect of NPY on the phosphorylation of the 87-kDa protein.

METHODS

Vas deferens were divided into three segments; proximal, middle and distal, and prelabelled in aerated Krebs buffer containing ^{32}-P (1 mCi/ml) for 60 min. NPY (500 nM) was added for 1–3 min, and the tissue was frozen on liquid N_2 and sonicated in boiling 1% SDS. The 87-kDa protein was immunoprecipitated with 87-kDa antibody and SAC cells. The immunoprecipitate was subjected to SDS-PAGE and autoradiography to quantitate the amount of incorporated ^{32}-P.

RESULTS

In the proximal segment of vas deferens, NPY inhibited 87-kDa phosphorylation. In the middle and distal segments, the effect was variable. The degree of inhibition in the proximal segment was 46 ± 7 % of control (FIG. 1). In preliminary experiments, the inhibition could be abolished by addition of pertussis toxin (100 ng/ml).

CONCLUSION

The results indicate that NPY-receptor activation in the proximal vas deferens leads to inhibition of phosphorylation of 87-kDa protein. The 87-kDa protein has shown to be phosphorylated and translocated from membranes to soluble phase during depolarization-induced Ca^{2+} influx in synaptosomes.[3] NPY was found to presynaptically inhibit nor-

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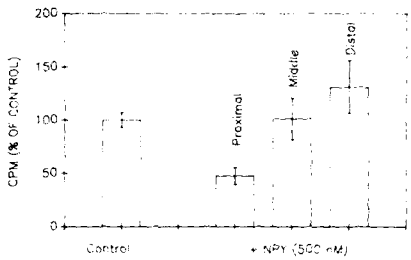

FIGURE 1. Effect of NPY (500 nM) on phosphorylation of 87-kDa protein in proximal, middle and distal part of rat vas deferens expressed as per cent change (± SEM, n = 4) from control.

adrenaline release in vas deferens,[4] an effect later shown to be confined only to the proximal half.[5] NPY was also found to inhibit cAMP accumulation in vas deferens[6] and to inhibit cardiac adenylate cyclase through a pertussis toxin sensitive G-protein.[7] It may be speculated that the observed effects of NPY on 87-kDa phosphorylation could involve crosstalk between an adenylate cyclase and a protein kinase C in the vas deferens, and that it also may be related to presynaptic inhibition of noradrenaline release.

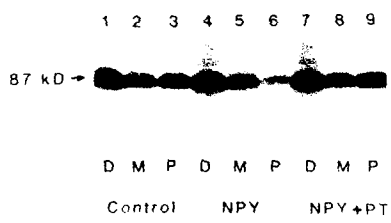

FIGURE 2. Effect of pertussis toxin on NPY-induced inhibition of 87-kDa protein in rat vas deferens. Autoradiograph shows 32-P-labelled 87-kDa protein that was immunoprecipitated using 87-kDa antibody, and separated on SDS-PAGE. *Lanes 1–3:* control, **lanes 4–6:** NPY (500 nM), *lanes 7–9:* NPY (500 nM) + pertussis toxin (100 ng/ml). *Lanes 1,4,7* are from distal (= epididymal), *lanes 2,5,8* from middle, and *lanes 3,6,9* from proximal (= prostatic) vas deferens.

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Cardiac Properties and Conformational Studies of Neuropeptide Y (NPY) and NPY(17–36)[a]

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The presence of neuropeptide Y (NPY) in high concentrations in nerve fibers surrounding blood vessels and heart has led to numerous investigations of the cardiovascular effects of this hormone. These studies have shown that NPY inhibits the contractile force[1,2] and the adenylate cyclase activity[3] of cardiac muscles and myocytes, respectively. Furthermore, specific receptors of NPY in the rat cardiac ventricular membranes have been characterized recently in our laboratory.[4] In the present study we investigated the effects of NPY and NPY(17–36) on ^{125}I-NPY binding and adenylate cyclase activity of rat cardiac ventricular membranes. We also compared the solution structures of these peptides by 2D NMR.

NPY and NPY(17–36) were synthesized by solid phase method and purified by reversed phase chromatography. Radioreceptor assay investigations revealed that NPY(17–36) (IC_{50} = 2 nM) exhibits nearly equal potency as NPY (IC_{50} = 0.5 nM) in displacing the ^{125}I-NPY bound to ventricular membranes. Furthermore, as previously reported,[3] NPY inhibited both basal and isoproterenol-stimulated cardiac adenylate cyclase activity in a dose-dependent manner. In contrast NPY(17–36) stimulated the basal and enhanced the isoproterenol-stimulated cardiac adenylate cyclase activity in a dose-dependent way (FIG. 1). However, other COOH-terminal fragments, 13–36, 16–36, 19–36, 20–36 and 25–36, inhibited isoproterenol-stimulated adenylate cyclase activity in the same way as NPY. Pertussis toxin treatment blocked the inhibitory effect of NPY, but had no effect on the stimulatory adenylate cyclase activity of NPY(17–36). Moreover, NPY(17–36) (1 μM) shifted the inhibitory dose-response curve of NPY on cardiac adenylate cyclase activity (IC_{50} = 4 nM) to the right (IC_{50} = 158 nM).

Solution conformations of NPY and NPY(17–36) were studied in DMSO by 2D NMR.[5] NPY manifested a rich NOE spectrum. The prominent features of this spectrum are a network of cross-peaks between N_iH and $N_{i+3}H$ protons of Ala_{18}-Tyr_{36} region which are clearly indicative of α-helical structures in the COOH-terminal region. A

[a]Supported in part by United States Public Health Service Grants GM 38601 (A. B.) and CA 48057 (W.T.C.)

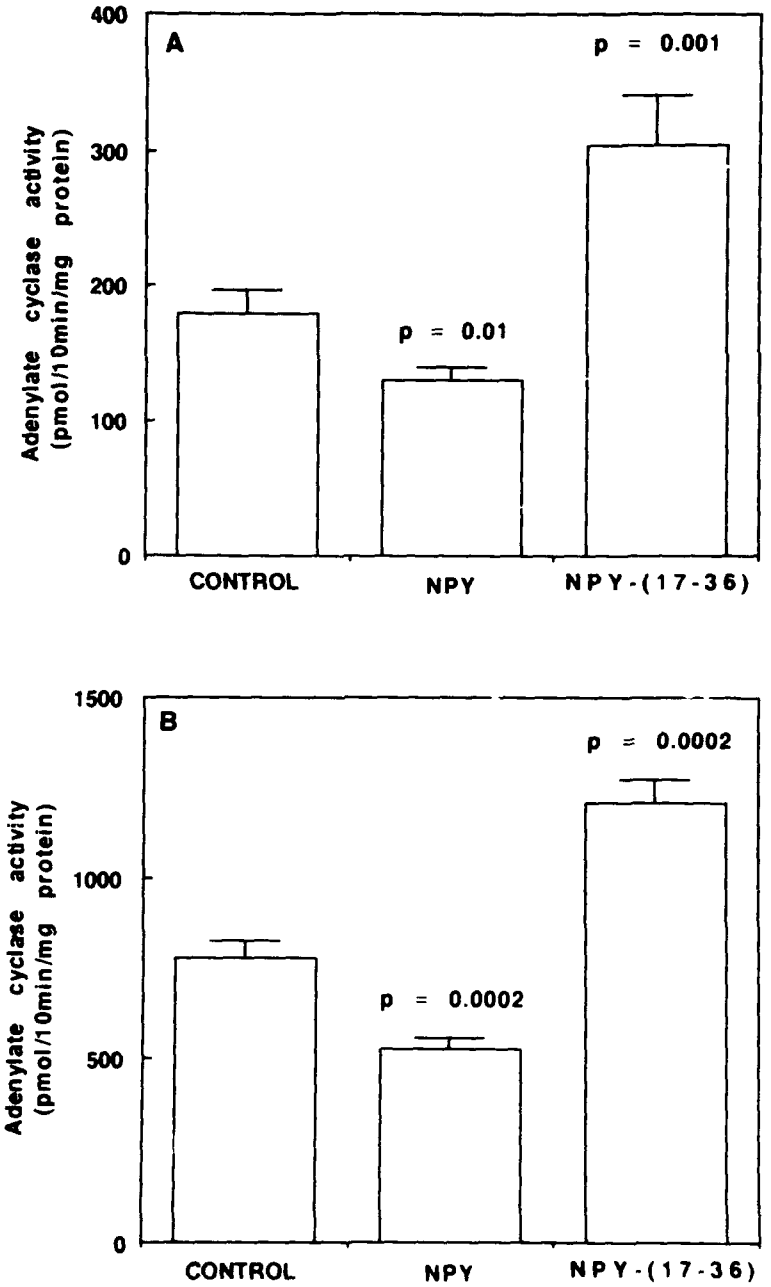

FIGURE 1. Comparison of the effects of NPY (1 μM) and NPY(17–36) (1 μM) on the basal (**A**) and isoproterenol-stimulated (**B**) adenylate cyclase activity of rat cardiac ventricular membranes.

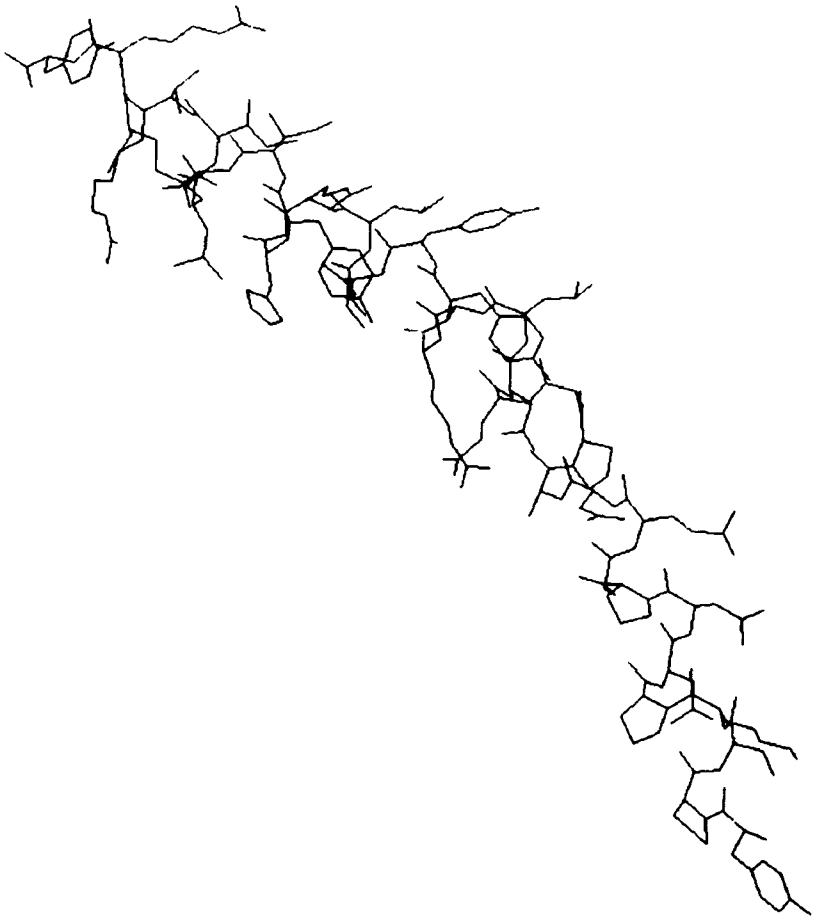

FIGURE 2. Minimized structure of NPY satisfying NOE constraints.

similar network of cross-peaks was also present in the NOE spectrum of NPY(17–36). In addition, the existence of short proton-proton contacts in the NH_2-terminal region, indicative of β-turns, were also evident in the NOE spectrum of NPY. These observations corroborate the evidence previously obtained from CD and Raman studies[6] and have formed the basis for molecular mechanics-dynamics studies (FIG. 2).

The results presented in this paper suggest that NPY(17–36) interacts with NPY receptor with high affinity, but fails to transduce the signal to the G_i protein. However, it stimulates adenylate cyclase activity probably by interacting with another receptor coupled to a G_s protein. These two factors may, in concert, antagonize the inhibitory adenylate cyclase activity of NPY. Furthermore, these observations suggest that COOH-terminal α-helical region of NPY is crucial for binding to the cardiac receptors.

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Structure-Activity Relationships of Biologically Active Analogs and Fragments of Neuropeptide Y[a]

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INTRODUCTION

The aims of our groups have included the elucidation of structure-activity relationships (SAR) for neuropeptide Y (NPY), its analogs and fragments. To this end we have synthesized many analogs and fragments of NPY in an effort to determine which conformational motifs may be required for receptor selectivity and efficacy.[1,2] Herein we report a tentative correlation between biological activities derived from biological assay data[3] and circular dichroic spectra of analogs reported for the first time in an accompanying communication.[4] Methods are to be found in REFERENCES 1 and 2.

RESULTS AND DISCUSSION

Of the many analogs and fragments of NPY_{18-36} that were synthesized, N-terminally modified analogs, analogs in which D-substitutions were made in the central region of the molecule, and C-terminally modified analogs were studied.

Modifications to the N-terminal region of NPY_{18-36}, *i.e.*, N-acetylation, N-methylation, desamination of Ala^{18} or D-substitution of either Ala^{18} or Arg^{19} resulted in peptides with no measurable hypotensive activity, while analogs in which D-substitutions were made in more central positions showed significant hypotensive activity. C-termi-

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nally modified analogs similarly retained potencies of the same order as that of NPY_{18-36} with the notable exception of $NPY_{18-36}OH$ which was inactive. Also inactive in this assay were the C-terminally deleted analogs NPY_{18-24}, NPY_{18-25}, NPY_{18-27} and NPY_{18-32}.[4] The most potent analog tested was $[DTyr^{21}]NPY_{18-36}$ (TABLE 1).

These findings indicated that the presence of diminished positive charge at the N-terminus (*e.g.*, N-acetyl-NPY_{18-36}) resulted in compounds which were inactive as were analogs in which substantial negative charge resided at the C-terminus (*e.g.*, $NPY_{18-36}OH$). Based upon the macrodipole stabilization of α-helices proposed by Shoemaker *et al.*[5] and Fairman *et al.*[6] we hypothesized that the presence of an α-helical region at the N-terminus of these analogs was detrimental to biological activity while the presence of an α-helical domain at the C-terminus was required for activity.

In order to test this hypothesis we examined the CD spectra of the more significant analogs, *i.e.*, NPY_{18-36}, $NPY_{18-36}OH$ and $[DTyr^{21}]NPY_{18-36}$ (TABLE 1). NPY_{18-36} was not found to be α-helical in 100% phosphate buffer but readily developed an α-helical domain in 15% TFE-phosphate buffer. $NPY_{18-36}OH$ however did not assume an α-helical conformation even in high concentrations of TFE (80%) consistent with our hypothesis. It was also expected that $[DTyr^{21}]NPY_{18-36}$ would exhibit α-helicity either in pure phosphate buffer or in relatively low concentrations of TFE. It was found, however, that α-helicity could not be induced in this compound in pH 8 phosphate buffer and different concentrations of TFE, suggesting that α-helicity as determined by CD is not a strict requirement for biological activity.

TABLE 1. Biological and CD Data for Selected NPY_{18-36} Analogs[a]

Compound	ΔMAP (mmHg) at 5 Mins (0.4 mg/kg)	Molar Ellipticity $\times 10^{-4}$ deg. cm^2 $dmole^{-1}$
NPY_{18-36}	−28 ± 5	−1.9 and −1.8[b]
$NPY_{18-36}OH$	−5 ± 5	−0.8[c]
$[DTyr^{21}]NPY_{18-36}$	−52 ± 5	−1.4[c]

[a]Concentration of samples was 1 mg ml^{-1}.
[b]At 208 and 222 nm.
[c]At 197 nm.

CONCLUSIONS

From the above data it is clear that while the presence of a random coil domain at the N-terminus of the compounds studied is compatible with biological activity, so is the presence of an α-helical region in the C-terminus. In fact, the substantially diminished potencies observed for analogs in which D-substitutions have been made in the C-terminus suggest that a well defined secondary structural motif is required in this region of NPY_{18-36}. In order to more fully explore this assertion, further conformational analysis of NPY_{18-36} has been undertaken employing 500 MHz proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and computer simulations. From these studies a compact structure has been proposed.[7]

ACKNOWLEDGMENTS

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Muscular Neuropeptide Y Receptors Involved in the Potentiation of the Noradrenaline-Induced Vasoconstriction in Isolated Coronary Arteries[a]

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The finding that NPY is co-stored with noradrenaline (NA) in nerves surrounding blood vessels and that these substances are co-released from sympathetic nerves, poses the attactive hypothesis that NPY and NA play a concerted role in the regulation of the vascular wall. Following NPY administration, the vascular system becomes supersensitive to subsequent applications of catecholamines.[1] *In vitro* experiments show that preincubation with NPY potentiates the contractile action of catecholamines and other pressor agents in a variety of blood vessels.[2,3] To further understand the physiology of NPY and NA in the coronary vasomotor tone, we designed *in vitro* experiments where NPY and NA are co-administered to superfused coronary arteries mimicking the action of sympathetic stimulation. Experiments were aimed at clarifying whether the NPY-induced potentiation of the NA contractions is receptor mediated and to evaluate whether the vascular endothelium is involved in the NPY-induced potentiation.

Helical strips of circumflex or descendent dog coronary arteries (15–20 mm) were prepared to record isometric muscular tension;[3] tissues were superfused with Krebs-Ringer buffer gassed with 95% O_2/5% CO_2. Noncumulative concentration-response

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curves were performed adding NA to the superfusion buffer in the absence or following a 5 min tissue preincubation with 0.2 nM NPY, peptide fragments or PYY. The NPY-induced potentiation experiments were performed in intact or denuded arteries.

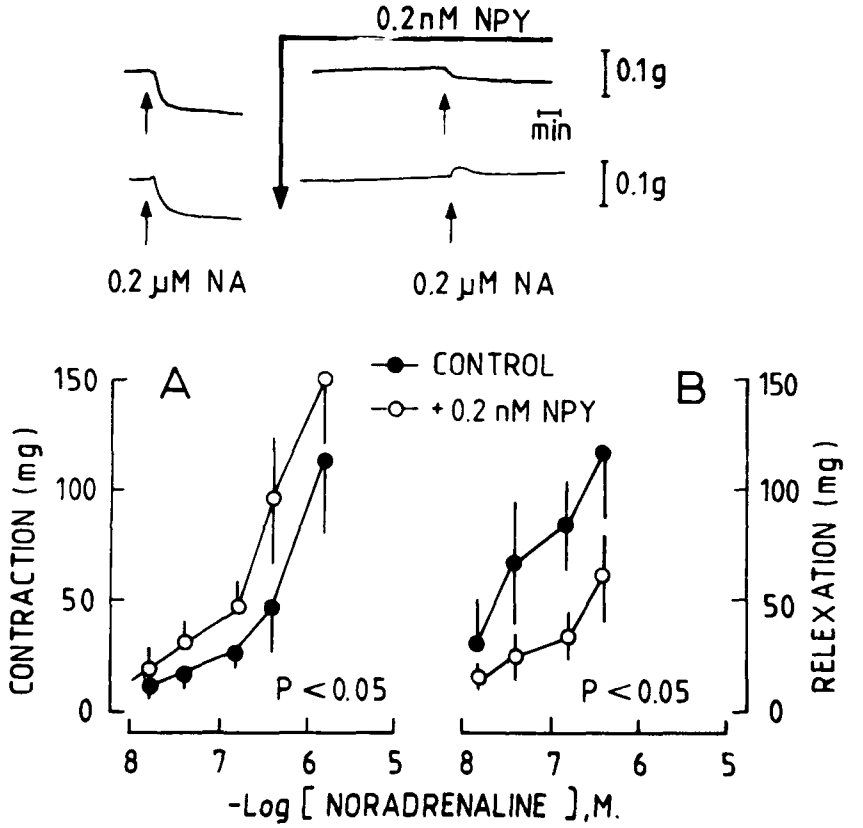

FIGURE 1. Effect of 0.2 nM NPY on the vasomotor actions of noradrenaline (NA) in helical coronary strips incubated in the absence of propranolol. *Polygraphic tracings:* Recording of representative cases obtained following the application of 0.2 μM NA prior to and after a 5 min tissue incubation with 0.2 nM NPY. Note that in one case NPY reduced the magnitude of the NA-induced coronary relaxation while in the other strip NPY reverted the NA-induced relaxation to a contractile response. *Lower panel:* (**A**) Synergism of the contractile action of NA by preincubation with 0.2 nM NPY ($n = 8$ separate strips, $p < 0.05$, analysis of variance); (**B**) Reduction of the NA-induced relaxation by preexposure to 0.2 nM NPY ($n = 7$, $p < 0.01$, analysis of variance). Symbols refer to mean values; bars to SE

NA (0.02–2 μM) caused either a relaxation (27 out of 50 separate strips) or a contraction (23/50) which in only 8/50 cases was sustained. In the rest of the strips, the contraction was followed by relaxation. The NA-induced contractions are concentration dependent and statistically potentiated by 0.2 nM NPY, concentration that did not significantly modify *per se* the tone (FIG. 1). When NA relaxed the vessels, NPY reduced

the magnitude of the relaxation displacing the NA concentration-response curve; in some occasions following 0.2 nM NPY, NA caused a transient contraction (FIG. 1). Upon addition of 1 μM propranolol to the incubation media, NA contracted the coronaries in a sustained fashion. That the potentiating action of NPY on the NA-induced contractile response is likely related to NPY receptor activation is supported by the fact that the NPY fragment 5-36 but not 11-36 mimicked NPY (FIG. 2). In addition, tissue incubation with PYY, significantly displaced the NA concentration-response curve to the left in about the same magnitude as NPY. When the synergism was examined at a fixed concentration of 70 nM NA (inset of FIG. 2), it is evident that the rank order of potency is NPY ≥ PYY ≥ NPY_{5-36} > NPY_{11-36}.

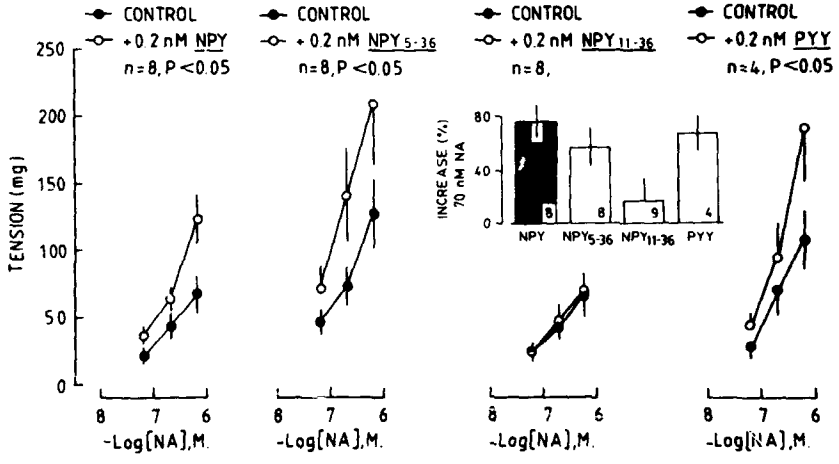

FIGURE 2. Noradrenaline (NA) contraction-response curves in tissues pretreated with 1 μM propranolol prior to and following incubation with NPY and related peptides. Separate helical coronary strips were used to record noncumulative NA responses prior to and following a 5-min incubation with either 0.2 nM NPY (8 strips), NPY_{5-36} (8 strips), NPY_{11-36} (8 strips) or PYY (4 strips). Symbols indicate mean values; bars SE NPY, NPY_{5-36} and PYY but not NPY_{11-36} caused a significant leftward displacement of the NA concentration-response curve ($p < 0.05$, analysis of variance). *Inset* shows the % increase in the muscular concentration caused by the application of 70 nM NA prior to and following a 5 min incubation with 0.2 nM NPY, peptide fragments or PYY.

Denuded arteries were supersensitive to the action of NA as compared to paired segments with intact endothelium. The absence of the endothelial layer did not interfere with the NPY-induced NA synergism; NPY caused in both cases a leftward displacement of the NA-response curve of about equal magnitude. The concentration of NA required to cause an increase of tension of 50 mg prior to NPY was 200 nM. This value decreased to 70 nM in the presence of NPY ($p < 0.05$) in denuded arteries. In the case of the intact arteries, these values were 300 and 90 nM NA ($p < 0.05$) respectively.

The present results can be interpreted as true synergism, the mechanism of which is as yet not clear. Our data supports the participation of NPY receptors likely localized on the vascular smooth muscle membrane. The synergism may be related to an increase in NPY receptor number or affinity, or to the facilitation of membrane transduction mechanisms. The implications of the concerted action of NPY and NA in the physiology of the coronary circulation in health and disease warrants further experimentation.

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Structural Study of the N-Terminal Segment of Neuropeptide-Tyrosine

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In order to better understand the contribution of the N-terminal residues of NPY to the receptor affinity and the expression of the biological activity, we synthesized a series of N-terminal analogues by solid-phase peptide synthesis, in which each of the first 10 amino acid residues was successively substituted by an L-alanine. The biological activities of these analogues were evaluated by using the rat vas deferens bioassay, and their affinity to CNS receptors was estimated by using the rat brain membrane binding preparation.

RESULTS AND DISCUSSION

NPY and analogues were synthesized using the BOP reagent,[1] according to synthetic methods recently described by Fournier *et al.*[2,3] All couplings, including those of Boc-asparagine and Boc-histidine(tosyl) were carried out with BOP. The quality of the material showed that a complete synthesis of NPY can be performed using only BOP as a coupling reagent, instead of the troublesome dicyclohexylcarbodiimide, as used by other groups.[4–7]

The rat vas deferens was chosen to evaluate the potency of each analogue. TABLE 1 shows the relative potencies of inhibition of the series [Ala[1]] to [Ala[10]] hNPY in the rat vas deferens. From these relative potencies, it clearly appears that positions 5 and 8, which are occupied by proline residues in the native molecule and are believed to contribute to the stability of the conformation by intramolecular hydrophobic interactions, are extremely sensitive to a substitution by L-Ala. This observation strongly suggests the importance of the integrity of the polyproline type II helix for the full expression of the biological activity in the vas deferens preparation.

TABLE 1 also gives the relative potencies of each analogue in inhibiting [^{3}H]-NPY

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specific binding. It appears that the proline residues located in position 2, 5 and 8 are also involved in the recognition mechanism of NPY by its CNS receptor. When the N-terminal tyrosine is substituted by an L-alanine residue, the potency of this analogue is considerably decreased, being approximately 40-fold less potent than hNPY. The substitution of the Tyr^1 by L-Ala would affect the conformation of NPY by decreasing the intramolecular stability. We believe that the NPY structure would contain a stacking of the aromatic nuclei of Tyr^1 and Tyr.[27] This stacking would contribute to the stabilization of the hairpin shape of the molecule, thus insuring a good recognition of the ligand by the receptor and the expression of the activity.

As shown in TABLE 1, there is a good correlation between the decrease of biological activity evaluated with the rat vas deferens bioassay and the decrease of affinity measured using the rat brain membrane preparation. Even if few residues seem to be more important in one assay than in the other, a condition suggesting that the brain and vas deferens NPY

TABLE 1. Relative Potencies in the Rat *Vas Deferens* Bioassay and Relative Affinities in the Rat Brain Membrane Binding Preparation of [ALA^{1-10}] hNPY Analogues

NPY Analogues	A: Rat *Vas Deferens* IC_{50}[a] ± SE[b] (nM)	R.P.[c]	B: Rat Brain Membrane Preparation IC_{50} ± SE[d] (nM)	R.A.[e]
hNPY	44 ± 2	100	4.1 ± 0.8	100
[ALA^1]hNPY	1698 ± 569	3	504 ± 152	0.8
[ALA^2]hNPY	651 ± 63	7	462 ± 93	0.9
[ALA^3]hNPY	227 ± 32	19	24 ± 6	17
[ALA^4]hNPY	554 ± 40	8	185 ± 21	2
[ALA^5]hNPY	1152 ± 13	4	1150 ± 266	0.4
[ALA^6]hNPY	155 ± 11	29	37 ± 8	11
[ALA^7]hNPY	303 ± 9	15	40 ± 8	10
[ALA^8]hNPY	1114 ± 231	4	2075 ± 408	0.2
[ALA^9]hNPY	681 ± 40	7	119 ± 13	4
[ALA^{10}]hNPY	981 ± 104	5	238 ± 83	2

[a] IC_{50} = the concentration of peptide producing a 50% inhibition of the maximum effect. Obtained from dose-response curves.

[b] SE = standard error (vas deferens bioassay, $n \geq 6$ and membrane preparation, $n \geq 3$).

[c] R.P. = relative potency.

[d] IC_{50} = the concentration of peptide producing a 50% inhibition of the maximum binding. Obtained from dose-response curves.

[e] R.A. = relative affinity.

receptors are different entities, although the brain might contain a heterogenous receptor population giving rise to a certain degree of overlap, it is clear that any changes involving the proline residues or tyrosine-1 are responsible for important decreases of affinity and activity. The substitution of residues Lys, Gly and Glu, at the position 4, 9 and 10 respectively, with L-alanine also presents nonnegligible reductions of potency and affinity.

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Investigations of the Structure-Activity Relationship of Neuropeptide Y[a]

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Neuropeptide Y (NPY), a 36 amino acid peptide isolated from the porcine brain,[1] is a well recognized neurotransmitter/neuromodulator both in the central and peripheral nervous system. We have demonstrated recently that NPY contracts in a dose-related fashion guinea pig airways[2] and rat colon by stimulating post- and prejunctionals receptors respectively. Using various fragments and analogues of NPY, the aims of this study were to characterize the NPY receptors responsible for these contractile effects and to determine if the two differently located biological receptors might correspond to the two types of NPY receptors described previously.[3]

METHODS

A series of C-terminal fragments of p-NPY and analogues of h-NPY with modifications of tyrosine residues in position 20 and 21 were synthesized by solid-phase peptide synthesis and purified by preparative HPLC. The homogeneity, purity and proper composition of the intended structures were confirmed by analytical HPLC and amino acid analysis. All synthetic peptides were tested for their biological activity in isolated guinea pig bronchus and rat colon by generating cumulative or noncumulative dose-response curves. Their relative potencies were evaluated using p- or h-NPY as standard.

RESULTS AND DISCUSSION

Comparison of results obtained with NPY fragments in both assay systems revealed that NPY receptors were differently sensitive to small changes introduced in the native molecule (FIG. 1). Deletion of only one amino acid such as Tyr^1, resulted in an almost inactive peptide in guinea pig bronchus (FIG. 1A) whereas it caused only a 5-fold decrease of NPY potency in rat colon (FIG. 1C). In this latter bioassay, the concentration-response (C/R) curve to NPY(2–36) exhibited parallelism to that of NPY but the maximal response averaged only 60% that of the entire molecule. Shorter NPY fragments up to (25-36),

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were proportionnally less potent and less active, exhibiting specially with fragments (16-36) to (25-36) nonparallel C/R curves. This differs from other studies in which NPY fragments (2-36) to (19-36) were reported as active as NPY, albeit less potent,[4,5] suggesting a difference in structural requirements for NPY receptors in various peripheral tissues. Another unexpected but interesting observation was the peculiar agonist property offered by NPY fragment (13-36). This fragment behaved similarly to NPY in rat colon (FIG. 1C) but appeared much more active (although less potent) than the mother molecule

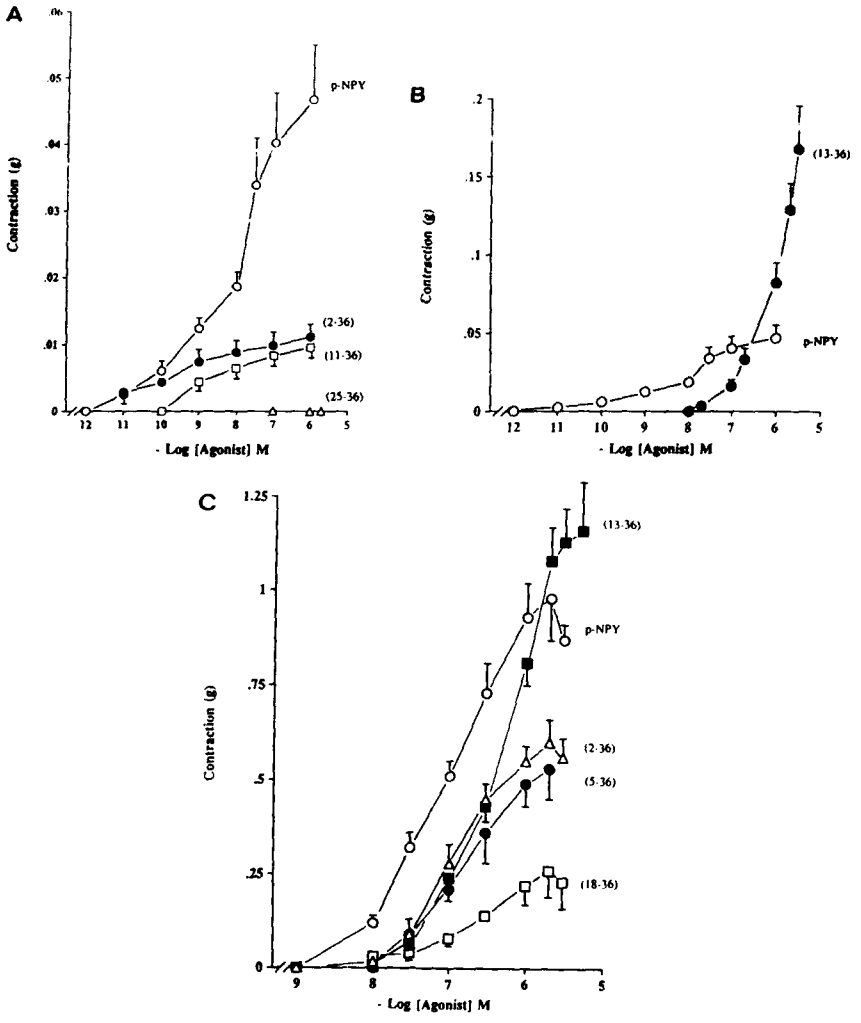

FIGURE 1. Concentration-response curves for NPY and NPY fragments in guinea pig bronchus (A,B) and rat colon (C). Each point and bar represent the mean and SE mean of data from 3 to 6 tissues. Results are presented as grams of contraction.

TABLE 1. EC_{50} Values, Relative Potencies (R.P.) and Developed Maximal Contractions (M.C.) for h-NPY Analogues in Isolated Rat Colon and Guinea Pig Bronchus[a]

Tissue	NPY Analogue	EC_{50} (*n*M)	R.P.	M.C. (g)
Rat colon	h-NPY	32.3 ± 11.3	100	0.73 ± 0.1
	[D-Tyr^{20}]	233 ± 33.3	13.8	0.60 ± 0.15
	[D-Tyr^{21}]	425 ± 25	7.6	0.54 ± 0.08
	[Trp^{20}]	125 ± 25	25.9	0.62 ± 0.08
	[Trp^{21}]	346 ± 57.6	9.6	0.41 ± 0.13
	[DTrp^{20}]	96.8 ± 35.4	33.3	0.61 ± 0.02
	[DTrp^{21}]	500 ± 100	6.4	0.6 ± 0.05
	[Phe^{20}]	225 ± 25	12.6	0.73 ± 0.11
	[Phe^{21}]	95.5 ± 19.1	33.8	0.98 ± 0.08
	[Cha^{20}]	233 ± 66	13.8	0.17 ± 0.06
	[Cha^{21}]	72.3 ± 14	44.6	0.67 ± 0.01
	[Tyr-O-Me^{20}]	85.7 ± 7.2	37.6	0.46 ± 0.1
	[Tyr-O-Me^{21}]	157 ± 71.7	20.5	0.8 ± 0.1
Guinea pig bronchus	h-NPY	2210 ± 1250	100	0.16 ± 0.02
	[Phe^{20}]	>10,000	—	0.12 ± 0.04
	[Phe^{21}]	>10,000	—	0.23 ± 0.03

[a]EC_{50}values are expressed as means ± SE mean of 3 to 7 determinations. Potency of h-NPY is arbitrarily fixed to 100%. In guinea pig bronchus, maximal contractions of h-NPY and its analogues were evaluated at a concentration of 10^{-6} M.

in guinea pig bronchus (FIG. 1B). Furthermore both peptides activated different mechanisms. The response to NPY was abolished by indomethacin (10^{-6} M) while that of NPY(13-36) was not. Such phenomenon did not occur in rat colon where contractile responses to both NPY and NPY(13-36) were totally inhibited in presence of atropine 10^{-6} M. Further evidence for the presence of different NPY receptor subtypes was provided with NPY analogues. As can be seen in TABLE 1, substitutions of tyrosine 20 or 21 were not detrimental for NPY receptor activation in rat colon. In agreement with previous *in vitro* binding and pharmacological studies,[6] despite showing small loss of potency, each analogue tested exhibited a relatively high level of biological activity. However, except for [Phe^{20}]- and [Phe^{21}]-NPY, none of the analogues studied here was able to induce significant contractile activity (up to 20% that of h-NPY) in guinea pig airways, even when tested in concentrations as high as 10^{-6} M. Taken together, these results support previous proposals of NPY receptors heterogeneity.[3,6]

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Pharmacological Actions of Neuropeptide Y and Peptide YY in Rat Colon[a]

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Neuropeptide Y (NPY) and peptide YY (PYY) are two related 36 amino acid polypeptides originally isolated from porcine brain and duodenum respectively.[1,2] Both peptides are present in the gastrointestinal tract, each having its own distinctive cell type and distribution.[3,4] Evidence is accumulating that these YY peptides are involved in the regulation of various gastrointestinal functions including neuronal control of motility. It has been proposed that NPY and PYY may interact with a common receptor to induce their actions.[5] The aims of this study were therefore 1) to examine and compare the *in vitro* effects of NPY and PYY on rat distal colon and 2) to establish whether the two peptides activate similar mechanisms.

METHODS

Segments of Sprague Dawley rat distal colon were set up for isometric recording (Grass Instruments) in a 5 ml organ bath (Tyrode solution) under a resting tension of 1 g. NPY or PYY was added to the baths in increasing concentrations, left in contact with the tissues until a steady level of tone was achieved, and then washed out. A minimum period of 20 min was allowed between doses challenge. When antagonists were used, they were added at least 15 min before the addition of the peptides. Noncumulative concentration-response curves to 5-HT and PgE_2 were also generated for comparison purposes.

RESULTS

NPY or PYY from 10^{-9} to 3×10^{-6} M produced a distinct concentration-dependant increase in the basal tone of the rat distal colon. The responses induced by NPY were characterized by an initial maximal peak which was followed by a progressively declining response and increased spontaneous activity (FIG. 1A). In contrast, PYY caused contractions which were short lasting; the initial peak being replaced by increased phasic activity

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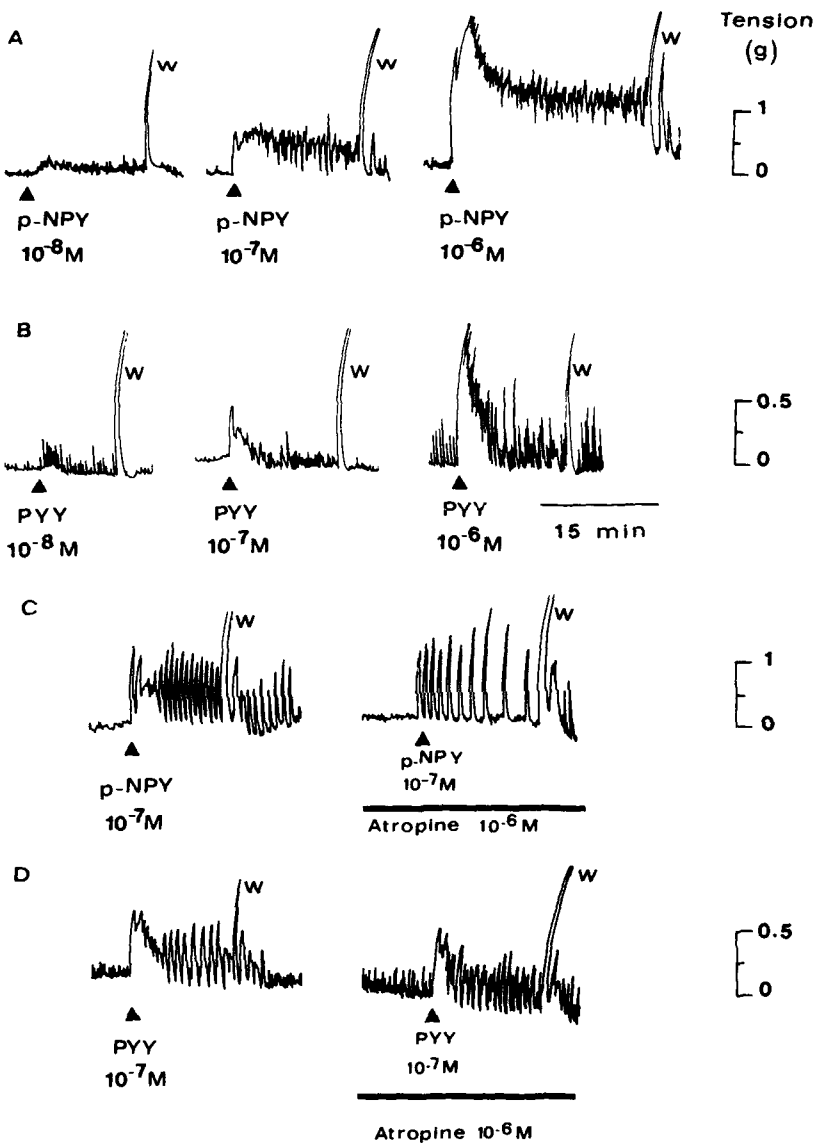

FIGURE 1. Contractile responses to NPY and PYY in rat distal colon. The original recordings presented here are from different tissue preparations. Panels **(A)** and **(B)** represent the effect of increasing concentration of NPY (A) and PYY (B) on the basal tone of rat colon. Panels **(C)** and **(D)** show tissue responses to single dose of NPY (C) and PYY (D) in absence and in presence of atropine (10^{-6} M).

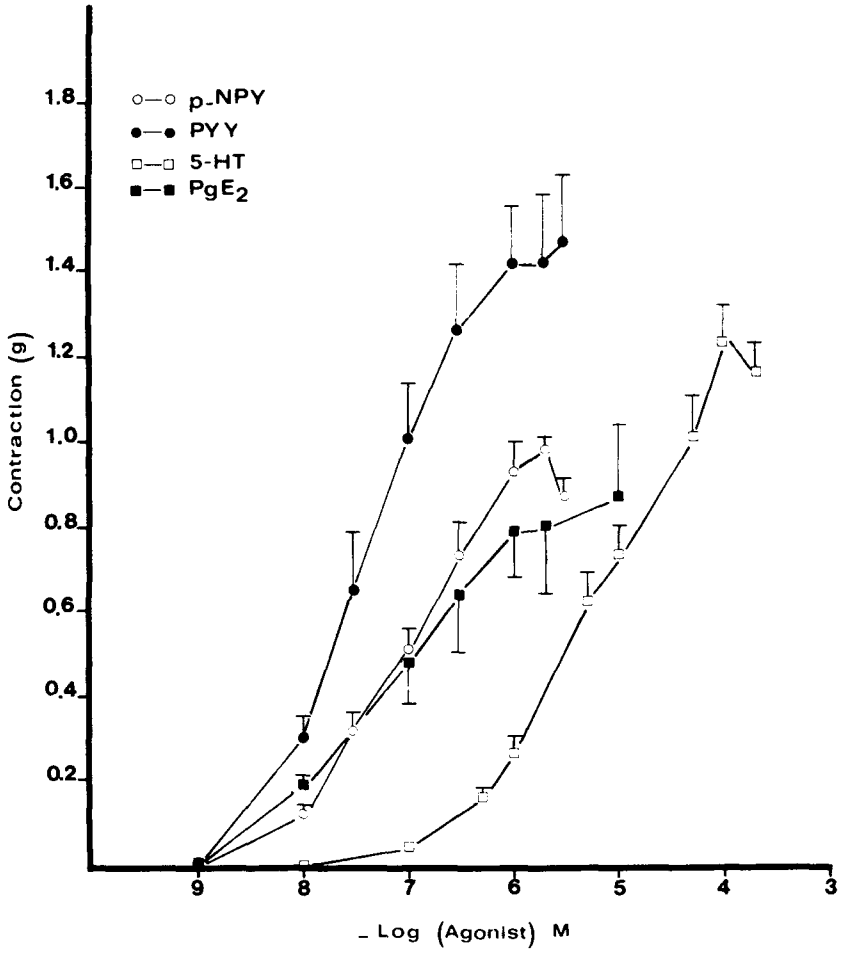

FIGURE 2. Concentration-response relationships for the contractile effects of NPY, PYY, 5-HT and PgE_2 on rat distal colon. Each point and bar represents the mean ± SEM of 6 to 10 determinations. Results are presented as grams of contraction.

after few minutes (FIG. 1B). The tonic responses to NPY were unaffected by methysergide, indomethacin, diphenhydramin or α and β blocking agents but were inhibited by atropine (10^{-6} M). In contrast, contractions elicited by PYY were resistant to the cholinergic antagonist (FIG. 1C.D). However, both NPY and PYY effects were totally abolished in the presence of tetrodotoxin (10^{-6} M). EC_{50} values for NPY and PYY were of the same order of magnitude, *e.g.*, 8.6 and 4.4 × 10^{-8} M respectively. On a molar basis PYY was equipotent to PgE_2 (EC_{50} = 5.9 × 10^{-8} M) but about 60 times more potent than 5-HT (EC_{50} = 2.6 × 10^{-6} M) in producing half maximal contraction. Furthermore, the maximal tension induced by PYY was found to be higher than those of other agonists (FIG. 2).

DISCUSSION

The present study showed that NPY and PYY have motor actions similar to those of local smooth muscle contracting factors present in the rat large intestine. This observation is consistent with the reported pattern of distribution of NPY-immunoreactive nerve fibers in muscle layers of the gut and with the localization of PYY in endocrine-like cells in distal colon.[3,4] Pharmacological analysis revealed that both NPY and PYY effects were neuronally mediated but via different neurotransmitters. On the basis of the present data, it seems likely that contraction of rat colon to NPY can be explained by the release of acetylcholine. The response to PYY however seems to involve a nonadrenergic and noncholinergic component; the nature of which remains to be determined. In conclusion, these findings support the concept of a role for NPY and PYY in the rat colon and suggest that these two related peptides exert their action at different operational receptor sites.

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Regulation of Neuropeptide Y Transcription and mRNA Levels by Nerve Growth Factor, Glucocorticoids, Cyclic AMP, and Phorbol Ester in PC12 Cells

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We are investigating the regulation of the preproneuropeptide Y gene (NPY gene) by hormones, second messenger systems, and factors that control cellular differentiation. We recently described[1] aspects of the regulation of the NPY mRNA level in PC12 rat pheochromocytoma cells. The abundance of NPY mRNA in these cells, which is very low under basal conditions, was markedly elevated by the synergistic actions of cAMP elevation and phorbol esters, which activate protein kinases A and C, respectively. In the present study we have examined by Northern blot analyses of mature NPY mRNA (700–800 bases) and by nuclear run-on transcription analyses the interactive effects of nerve growth factor (NGF) and glucocorticoids on NPY gene expression in PC12 cells highly sensitive to NGF.

Treatment of cells with 2.5 S NGF for 1–6 d increased the abundance of mature NPY mRNA (700–800 bases) 30–100-fold. The half-maximal and maximal effects of NGF were obtained with 16 and 65 ng/ml, respectively. Glucocorticoids such as dexamethasone (Dex), which alone generally elevated the NPY mRNA level slightly, potentiated by up to 3-fold the stimulation by NGF at early times (≤7 h) but strongly suppressed it at later times (≥25 h). The response to NGF (4–8 h) required ongoing protein synthesis, suggesting an initial induction by NGF of factor(s) acting on the NPY gene. In nuclear run-on analyses, treatment of cells for 1–24 h with NGF stimulated NPY gene transcription 4–8-fold, a response potentiated or inhibited by Dex during 2-h or 24-h treatments, respectively. Treatment with forskolin, which markedly elevates cAMP levels, or phorbol-12-myristate-13-acetate (PMA), which activates protein kinase C, elevated NPY mRNA levels 4–10-fold or ≤4-fold, respectively, while forskolin + PMA synergistically elevated NPY mRNA levels 30–70-fold and NPY gene transcription 6–25-fold. NGF + forskolin + PMA elevated NPY mRNA levels approximately 200-fold and NPY transcription 15–40-fold.

We conclude that the NPY gene is transcriptionally activated by NGF and by interactive actions of protein kinases A and C. The marked discrepancy between mRNA and apparent transcription rate elevations elicited by NGF suggests that NGF may elevate NPY mRNA by mechanism(s) in addition to transcriptional activation, such as mRNA processing and stabilization.

The striking NGF/glucocorticoid antagonism (≥24 h of treatment) affecting NPY gene regulation has been observed for other delayed inductions by NGF and may reflect

a mechanism for phenotypic choice during neural crest differentiation.[2] A possible molecular explanation is downregulation of NGF receptors by prolonged glucocorticoid treatment.[3]

This work is described in a full paper.[4]

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Strong Evolutionary Conservation of Neuropeptide Y between Mammals, Chicken, Goldfish, and Horned Shark

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The NPY family of peptides includes, in addition to NPY, also peptide YY (PYY) and pancreatic polypeptide (PP) as well as the fish pancreatic peptide PY. To investigate the degree of conservation of NPY at the structural level during evolution, as well as its relationship to the other members of this peptide family, we have isolated DNA clones encoding NPY from chicken, goldfish (*Carassius auratus*) and horned shark (*Heterodontus francisci*). We have previously reported that the chick and goldfish NPY sequences reveal a remarkable degree of homology, as they display only 1 and 5 differences, respectively, to human NPY.

Using a probe containing the entire coding region of a chick NPY cDNA clone, we have now screened a shark genomic library (kindly provided by Dr. G. Litman, Tampa Bay Research Institute, St. Petersburg, FL) under conditions of low stringency. Several phage clones were isolated and the cross-hybridizing region of one of these clones was subcloned in a plasmid vector and subjected to nucleotide sequence determination. The clone was found to contain exon two of the shark NPY gene.

Exon two of the NPY gene encodes the signal peptide and 34 of the 36 amino acids of mature NPY. The shark signal peptide differs considerably from the signal peptides of the other species, but the general hydrophobic character is maintained. In contrast, mature shark NPY displays only three differences to the human sequence. Two of these differences are conservative amino acid replacements; leucine instead of methionine and lysine instead of arginine.

Mature shark NPY has two differences to goldfish NPY. The shark sequence displays no unique amino acid residues as compared to the other known NPY sequences. This suggests that shark NPY may be identical to NPY of the common ancestor of cartilaginous fishes, bony fishes, and mammals.

In order to investigate the structural conservation of CPON, we are currently trying to localize exons 3 and 4 of the shark NPY gene by using low-stringency hybridizations.

The impressive similarity of shark NPY to human NPY makes this peptide one of the most highly conserved known (92% identity at the amino acid level), clearly more conserved than insulin. The strong sequence conservation of NPY indicates that this peptide subserves evolutionarily old and important functions.

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Cell-Type Specific Modification of Peptide Posttranslational Processing in Pituitary Cells[a]

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One approach to examine the peptide processing capabilities of a cell involves transfection of the cDNA for a foreign propeptide precursor into the cell and then detailed examination of the peptides produced by that cell. We have used that approach to examine the cell-type specificity of peptide processing in two widely used pituitary cell lines, mouse corticotropes (AtT-20) and rat somatomammotropes (GH_3). We have transfected the cell lines with vectors employing the mouse metallothionein-1 promoter to control expression of wildtype and mutant forms of human proNeuropeptide Y (proNPY) cDNA. The four types of proNPY cDNA, kindly provided by Dr. Jack Dixon (Purdue University), differ only in the pair of basic amino acids present at the single site of cleavage in the molecule.

When the four forms of proNPY cDNA were expressed in AtT-20 cells, a marked hierarchy of cleavage site preference was seen.[1] The wildtype -Lys-Arg-(KR) and the mutant -Arg-Arg- (RR) sequences were cleaved nearly to completion within a couple of hours. By comparison, the mutant RK-proNPY was cleaved much more slowly and less completely, and the mutant KK-proNPY was cleaved extremely poorly in the AtT-20 cells. When the corresponding four cell lines were created using the GH_3 cells as the host line, a very different result was obtained. The extent of cleavage of all four forms of proNPY proceeded approximately to the same extent, with the same kinetics.[1,2] After a 16-hour labeling in medium containing [^{3}H]leucine, medium samples were immunoprecipitated using an affinity-purified NPY antiserum and analyzed by SDS polyacrylamide gel electrophoresis (FIG. 1). A significant amount of cleavage of proNPY to NPY was seen with all four forms of proNPY as precursor. Peptide analyses demonstrated that the NPY-size immunoprecipitable molecules from both GH-NPY and AtT-NPY cell lines were authentic NPY.[1,2]

While the GH-NPY lines all cleaved proNPY to a certain extent (FIG. 1), the cleavage was not usually as complete as seen with the AtT-NPY cells. Since methods had been established to dramatically increase the number of secretory granules in the GH cells, using a treatment of estradiol, insulin, and epidermal growth factor in the medium,[3,4] we asked what effect such a treatment might have on the storage and processing of the exogenous proNPY in the transfected GH-NPY cells. As expected, the amount of growth hormone produced by the cells was reduced to one-third during the hormone treatment (FIG. 2A), while the level of the much more abundant prolactin rose four-fold (FIG. 2B). Similar hormone treatments had no effect on the ACTH production by transfected AtT-20

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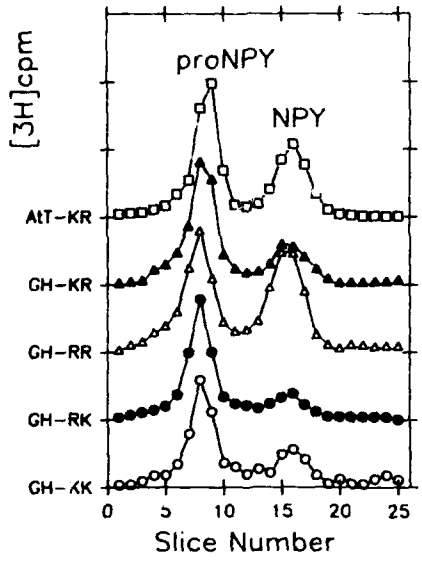

FIGURE 1. SDS-PAGE analysis of immunoprecipitated NPY-related peptides from pituitary cell lines. Cell lines: AtT-KR, AtT-20 cells which express -Lys-Arg-containing proNPY; GH-KR, GH_3 cells which express -Lys-Arg-containing proNPY; GH-RR, GH_3 cells which express -Arg-Arg-containing proNPY; GH-RK, GH_3 cells which express -Arg-Lys-containing proNPY; GH-KK, GH_3 cells which express -Lys-Lys-containing proNPY.

cells (FIG. 2C). During these changes in response to the hormonal treatment, the residence time of the proNPY-derived peptides increased strikingly in the GH_3 cells, with no change in the extent of cleavage of the proNPY. No effects were seen on proNPY storage or processing in the AtT-NPY cells.[2]

Thus, not only is the ability to process propeptide precursors cell-type specific, but also the changes in propeptide processing in response to external stimuli are also cell-type specific.

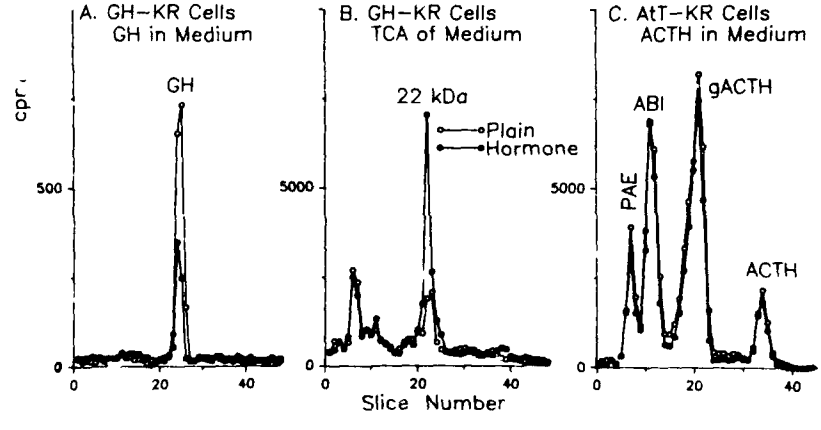

FIGURE 2. Effect of hormone treatment on endogenous peptide production in GH_3 cells and AtT-20 cells. Peptides analyzed were Growth Hormone (**A**) and Prolactin (**B**) from GH-KR cells, and ACTH (**C**) from AtT-KR cells.

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Novel Brain Specific Transcription Factors Form DNA-Protein Complexes with SP1 and TATA Regions of the Rat Neuropeptide Y Gene Promotor

Enhancement of Complex Formation by Nonneuronal Factors

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid peptide widely distributed throughout the mammalian central nervous system.[1,2] Its diverse functions are well documented.[3] Its role as a neurotransmitter or neuromodulator is well established. It is a vasoconstictor and coexpresses with adrenalin and certain neuropeptides in the brain. The access to genomic clones of human and rat NPY has enabled the elucidation of peptide structure and gene sequence and localized its mRNA distribution in neuronal and nonneuronal tissues.[4-6] However, the nature of binding sites for brain nuclear factors that form transcriptional complexes with the NPY gene is not known. The present study is conducted to gain new insights into transcriptional mechanisms of the rat NPY gene in brain and nonneuronal tissues. By a gel shift assay using [^{32}P]-end-labeled oligonucleotides annealed to the complementary strand, or Klenow-labeled enzyme restriction fragments, DNA sequences spanning -16 to -144 upstream of the transcriptional start site of the rat NPY promoter were analysed for putative transcriptional complexes formed by DNA-protein binding with nuclear extracts. Nuclear extracts were prepared from brain, spleen, liver and bone marrow obtained from adult Sprague Dawley rats.[7] DNA motifs were determined by methylation protection.[8]

RESULTS AND DISCUSSION

Using an oligonucleotide spanning -16 to -74 (oligo I), of the rat NPY promoter with "TATA-like" element located at -25,[5] nuclear extract from rat brain was found to form three major DNA-protein complexes, Y1, Y2 and Y3 showing binding affinities Y2>Y3>Y1 (FIG. 1A, panels 1 and 2). By comparison with complexes Y2 and Y3, lower affinity complexes Y4 and Y5 were detected with DNA sequences of an Xho I-Sma I restriction fragment spanning -78 to -144 (FIG. 1A, panel 3), or its related oligonucleotide, -75 to -133 (oligo II). No complexes were formed with sequences -142 to -201

(oligo III) (FIG. 1A, panel 4). Effective competition was achieved with unlabeled oligo I and oligo II, but not with oligo III or unrelated DNA (FIG. 1B), indicating specific binding with promoter. Methylation protection identified three distinct binding sites on the promoter for brain nuclear factors: a unique 18-mer sequence 5'-CTCCATAAAAGCCCGTTG, which includes "TATA-like element" ATAAAA at -25, and a novel 9-mer motif 5'-GCCCCTCCT located at -50, as well as its complementary

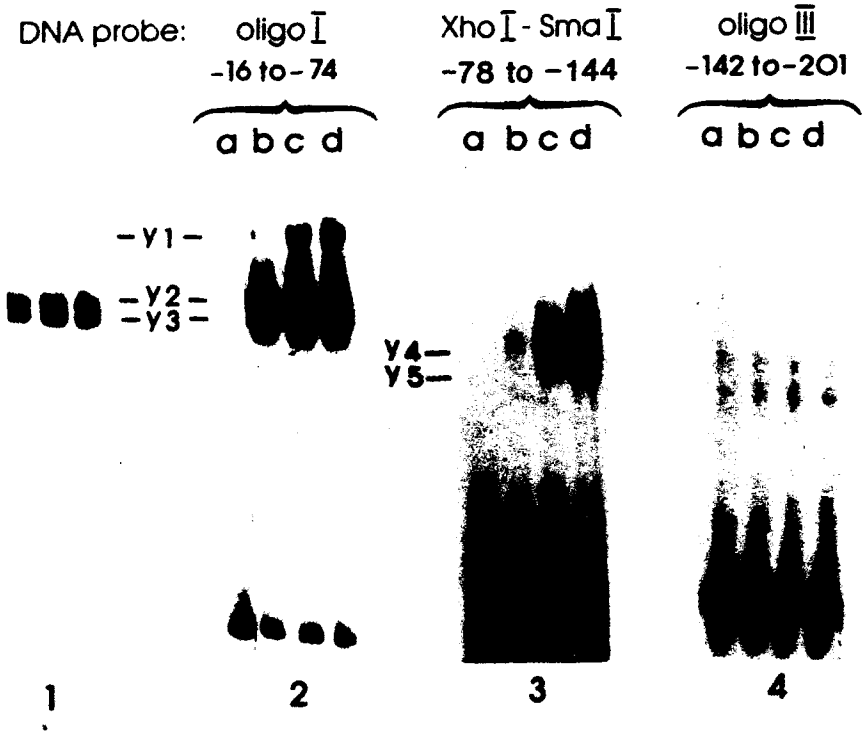

FIGURE 1A. Gel shift assay for DNA-protein complexes using nuclear extract incubated with [^{32}P] labeled double stranded oligonucleotides (oligo I or III) or restriction fragment Xho I-Sma I indicated. Binding to promoter as a function of concentration of brain nuclear extract protein is shown: (a) free probe without nuclear extract, (b) 8 μg; (c) 16 μg; and (e) 32 μg of brain nuclear protein (*panels 1 to 4*). The different concentrations of brain nuclear extract were incubated with approximately 0.5 to 1 ng of the indicated labeled probes in a binding assay at 30 degrees centigrade, in presence of 4 μg poly(di-dC). The resulting complexes were separated from unbound input fragment by electrophoresis on 6–8% polyacrylamide gel. *Panels 1 and 2* show results of autoradiography of same experiment; *panel 1*, after 12-hr exposure to Kodak X-ray film with intensifyying screen; *panels 2–4*, after 30 hours exposure.

sequence (FIG. 1C, 1). Altogether, the findings show recruitment of three distinct binding sites to assemble brain DNA-protein complexes.

Interestingly, the anti-sense 9-mer sequence resembles the consensus G/TGGCGGGGC/AAT, site for the general transcription factor Sp1. Additional experiments showed the ability of unlabeled 5'-GCCCCTCCT sequences to compete off brain specific DNA-protein complexes from labeled oligo I with 100% efficiency in contrast to

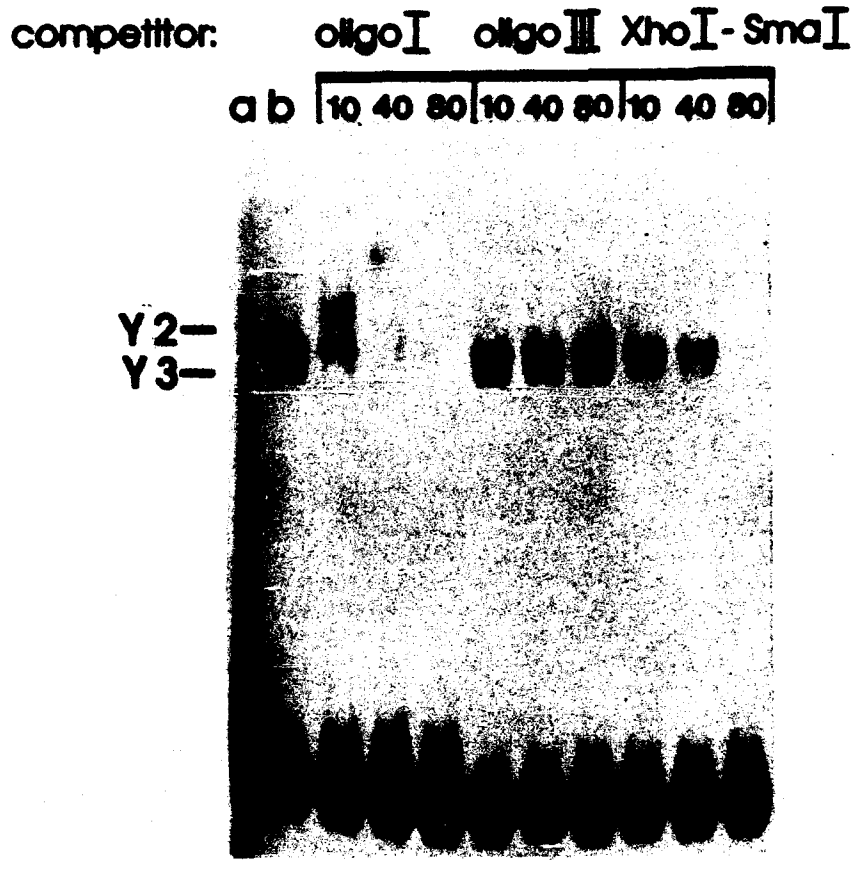

FIGURE 1B. Competition to identify promoter specificity by the addition of unlabeled oligonucleotide to displace [^{32}P]-labeled oligo III bound in DNA-protein complexes in similar gel shift assays. Just before addition of labeled probe, competitor DNAs were added in the ng quantities indicated. Formation of DNA-protein complexes were monitored by gel shift assays as in FIGURE 1A. (a) No extract added; (b) 10 μg of brain nuclear extract in absence of any competitor.

50% competition effected by its complementary strand (FIG. 1C, 2). The findings strongly suggest a key role for the 9-mer motif identifying it as a major sequence that assembles specific DNA-protein complexes with brain nuclear factors; cooperation with TATA-like and Sp1 sites on the promoter bind brain nuclear factors, forming transcriptional complexes to govern NPY gene expression in brain. Addition of a spleen, liver or bone marrow nuclear extract to a limited amount of brain nuclear extract enhanced formation of brain complexes (FIG. 2A), whereas each nonneuronal extract formed its individual profile of DNA-protein complexes with electrophoretic characteristics different from brain complexes (FIG. 2B). The findings suggest recruitment of tissue specific mechanisms by the NPY promoter and synergism between neuronal and nonneuronal factors.

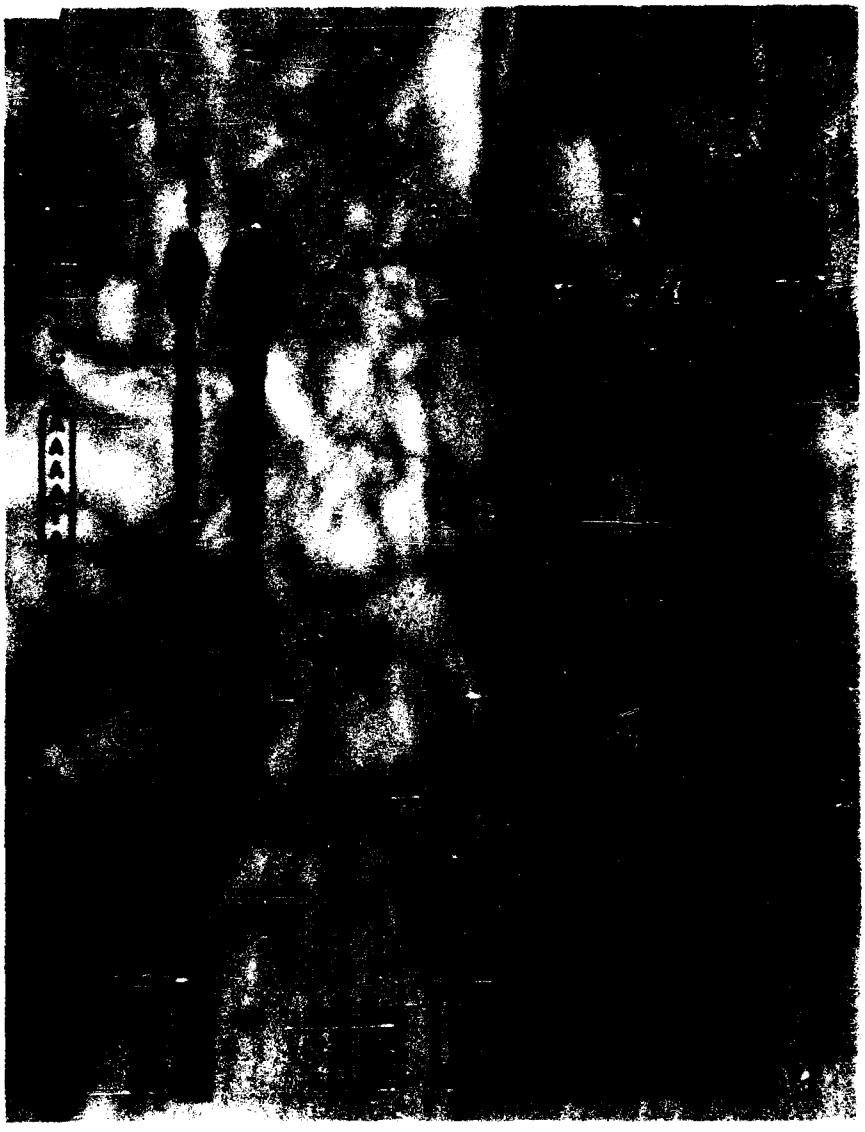

FIGURE 1C. *Panel 1.* Methylation protection of oligonucleotide I using total brain nuclear extract. *The DMS protection was done by incubation of a total brain extract with* [^{32}P]-*labeled coding strand* of oligonucleotide I. The guanine reaction of the free probe (oligonucleotide I) is indicated (unbound);[1] the bound (DNA-protein complex) guanine reacted probe is shown. *Panel 2.* Competition analyses using a 9-mer oligonucleotide, 5′ -GCCCCTCCT. Competitive binding was done as in FIGURE 1B, using [^{32}P]-labeled oligonucleotide I and brain nuclear extract. The quantities in ng are shown of a 9-mer oligonucleotide, 5'-GCCCCTCCT as competitor. The competitor oligonucleotide is located at -50 of the coding strand. DNA-protein complexes were assayed as described in FIGURE 1B. (a) No extract added; (b) 10 μg of brain nuclear extract in the absence of competitor.

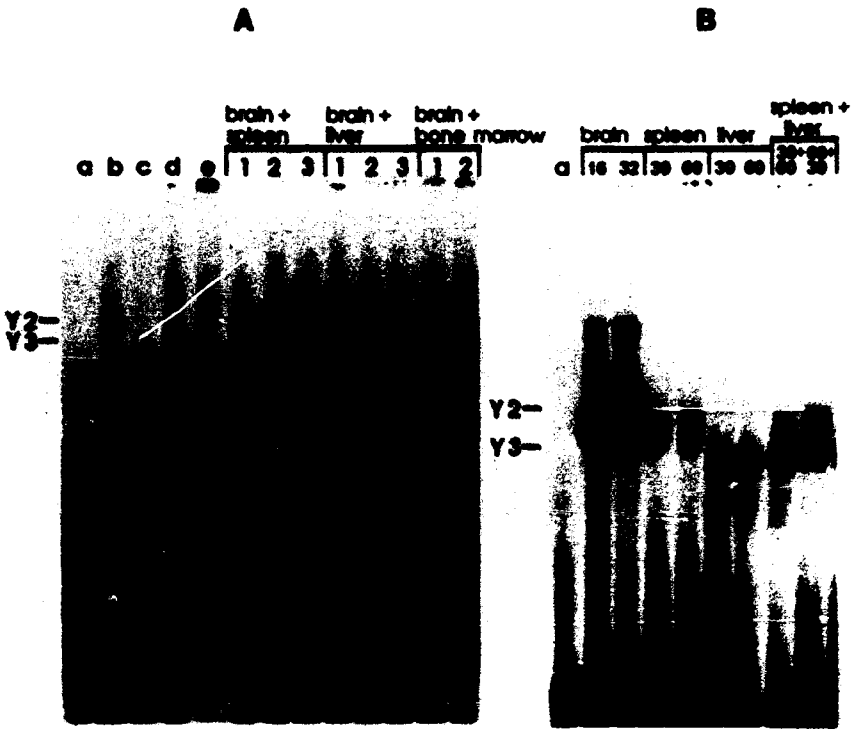

FIGURE 2. **(A)** Enhancement of brain DNA-protein complexes by nonneuronal factors. Mixing experiments were done by adding increasing amounts of a nonneuronal extract to a limiting amount (5 μg) of a brain nuclear extract and the mixture was incubated with [^{32}P]-labeled oligonucleotide I. Complexes formed were assayed by 3–12% gel gradient. (a) Probe alone; (b) brain, 5 μg; (c) spleen, 60 μg; (d) liver, 60 μg; and (e) bone marrow; each nonneuronal extract was added to 5 μg of a brain extract at (1) 30 μg, (2) 60 μg and (3) 120 μg of the indicated nonneuronal extract. **(B)** Enhancement of brain DNA-protein complexes using nonneuronal extract mixed with one another and bound with labeled oligo I. The individual electrophoretic mobilities of complexes formed with each nonneuronal extract was observed by gel gradients, with features different from brain specific DNA-protein complexes.

CONCLUSIONS

In summary, the rat NPY promoter forms two high affinity brain specific DNA-protein complexes Y2 and Y3, with brain nuclear factors. Being the major high affinity complexes with distinct features, they most likely constitute brain specific transcription complexes. Brain DNA-protein complex formation is enhanced by nonneuronal factors indicating synergistic cooperation. The study has identified a novel 9-mer core motif, 5′ -GCCCCTCCT at -50, which binds brain nuclear factors and by cooperation with Sp1 (5′ -AGGAGGGGC) and TATA (5′ -CTCCATAAAAGCCCGTTG) regions on the promoter form specific transcriptional complexes to govern NPY gene expression in brain.

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Defect of Binding of Nuclear Factors from Embryonic and Newborn Brain Nuclear Extracts from Autoimmune NZB Mice with the Rat Neuropeptide Y Gene Promoter

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INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid peptide with neurotransmitter, neuromodulator and vasoconstrictor functions. It is distributed throughout the mammalian central nervous system.[1-3] Its diverse functions are well established.[3] The access to genomic clones of human and rat NPY has enabled the elucidation of gene sequence and peptide structure[4-6] and has localized its mRNA distribution in neuronal and nonneuronal tissues confirming the broad functions of NPY.[7] Studies using complementary DNA (cDNA) of the rat NPY gene have shown increased levels of NPY mRNA in megakaryocytes of certain mouse strains like the New Zealand Black (NZB) mouse, that develop a spontaneous form of autoimmune disease resembling human systemic lupus erythematosus.[3] The mechanisms of increased expression are not known. The present study is conducted to define transcriptional mechanisms of the NPY gene in the autoimmune NZB mouse which manifests serological disease by 2 weeks of age. By a gel shift assay using [^{32}P]-end-labeled oligonucleotides annealed to the complementary strand, DNA sequences spanning -16 to -133 upstream of the transcriptional start site of the rat NPY promoter were analyzed for putative transcriptional complexes formed by DNA-protein binding with nuclear extracts.[7] Nuclear extracts were obtained from NZB mice and compared with normal C57Bl/6J mouse strains in different developmental stages: (a) embryos at 8 days gestation, (b) brain from newborn mice at 1 day of age (P1) and (c) at 6 weeks.

RESULTS AND DISCUSSION

Using an oligonucleotide spanning -16 to -74 (oligo I) of the rat NPY promoter with a "TATA-like" element located at -25, nuclear extract from embryos of C57Bl/6J mice at 8 days gestation was found to form a major complex YM (FIG. 1A: b,c). Interestingly, two major complexes YM1 and YM2 were formed with nuclear extract from brain of newborn C57Bl/6J, at 1 day after birth (FIG. 1B: a,b). The same profile of complexes YM1 and YM2 showing higher binding affinity was obtained using brain nuclear extract from 6-week-old C57Bl/6J mice (FIG. 1C: a). Interestingly, nuclear extract from 8th day embryos of NZB mice did not form complex YM; instead a different complex YZ was

formed (FIG. 1A: d,e). Moreover, brain nuclear extract from newborn NZB mice also showed a defect in formation of complexes YM1 and YM2 detected in brain of C57Bl/6J newborn mice. However, brain nuclear extract from 6-week-old NZB mice formed complexes YM1 and YM2 with binding affinity and features comparable to those obtained with extract from 6-week-old C57Bl/6J mice (FIG. 1C: b). The distinct profiles of DNA-protein complex formation in development in the mouse suggest a strong influence of

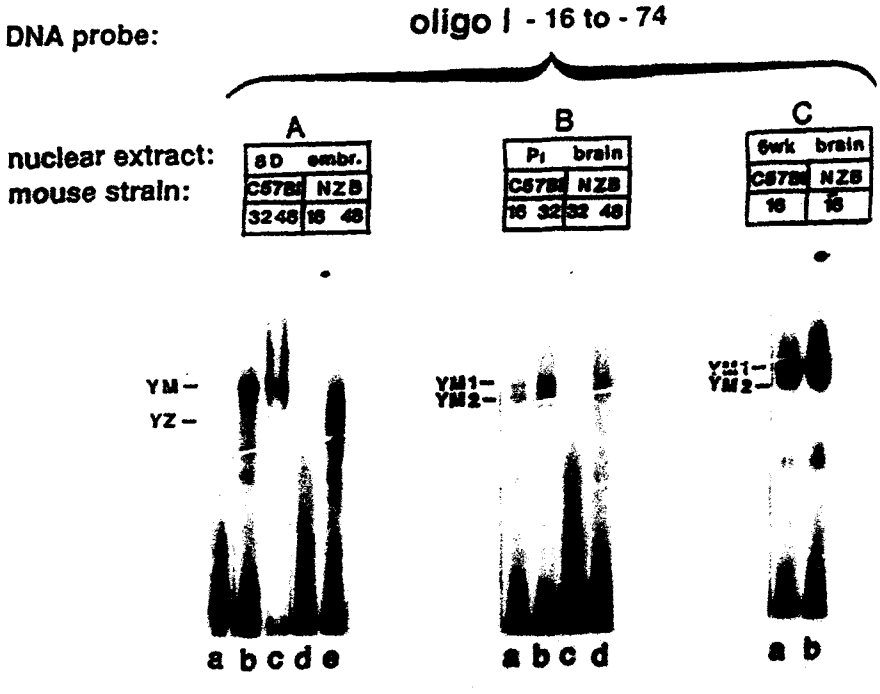

FIGURE 1. Gel shift assay for DNA-protein complexes using nuclear extract (n.e.) incubated with [^{32}P]-labeled double stranded oligonucleotides (oligo I and II). Nuclear extracts (n.e.) were obtained from two mouse strains and compared for formation of putative transcriptional complexes in ontogeny. Oligo I -16 to -74, was used at approximately 0.5 to 1.0 ng used in nuclear extract binding as described. Complexes formed were separated from remaining unbound input of labeled probe by electrophoresis on 7% polyacrylamide gel electrophoresis. *Panel A:* (a) free probe without extract n.e.; (b) n.e. from C57Bl/6J embryo of 8 days gestation incubated with oligo I at (b) 32 μg, (c) 48 μg; and from NZB embryo (d) 16 μg, (e) 48 μg. *Panel B:* n.e. from newborn C57Bl/6J brain, (a) 16 μg, (b) 48 μg. Comparison with NZB newborn brain n.e. at higher concentration (c), 32 and (d) 48 μg. *Panel C:* brain n.e. from 6 week C57B/6J mice (a) 16 μg and (b) from 6 week NZB mouse, 16 μg.

developmental processes on mechanisms of DNA-protein binding in DNA-protein complex formation and thus govern gene expression. With an annealed [^{32}P]-labeled oligonucleotide -75 to -133 upstream of the promoter, similar nuclear extract from embryos of the C57Bl/6J mouse formed a different profile of complexes YM3 and YM4 (FIG. 2: b,c), whereas with brain extract from newborn C57Bl/6J mouse, a different complex YM5 was formed in addition to formation YM3 (FIG. 2: d,e). Complex YM3 and YM4 showed high

binding affinity using brain nuclear extract from the C57Bl/6J mouse at six weeks (FIG. 2: f). The increased affinity of DNA-protein complexes observed was apparent as the C57Bl/6J mouse advanced in development. By contrast, complexes YM3, YM4, and YM5 were barely detected in comparable extract from the NZB mouse in either embryonic nuclear extract (FIG. 2: g,h) or nuclear extract from brain of the newborn NZB mouse (FIG. 2: i,j). However, brain nuclear extract from the NZB mouse at 6 weeks formed complexes YM3 and YM4 with affinity and features comparable to those detected for normal C57Bl/6J mouse of same age (FIG. 2: k and f, respectively). The distinct profiles detected in this region of the promoter in development indicate that developmental factors regulate transcription by affecting DNA-protein complex formation. In the NZB mouse a defect in DNA promoter binding is detected affecting DNA-protein formation by nuclear factors with sites distant from the "TATA-like" region of the NPY promoter.

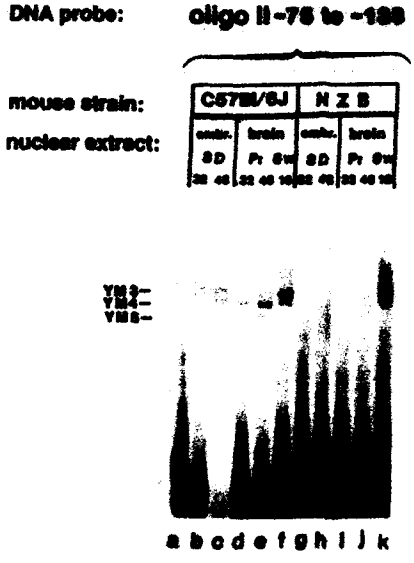

FIGURE 2. Comparison of binding using [^{32}P]-labeled oligo II with nuclear extracts from the same developmental stages as studied in FIGURE 1. (a) free oligo II without n.e.; oligo II incubated with C57Bl/6J n.e. from 8 day embryo, (b) 32 and (c) 48 μg; newborn brain, (d) 32 μg and (e) 48 μg, and 6 weeks brain, (f) 16 μg. Oligo II incubated with n.e. from NZB mice: embryo (g) 32 and (h) 48 μg; newborn brain (i) 32 μg and (j) 48 μg and (k), from 6 weeks, 16 μg.

CONCLUSIONS

Nuclear factors in ontogeny of the C57Bl/6J mice form DNA-protein complexes distinct from DNA-protein complexes formed by nuclear extract in later development at six weeks. The findings suggest distinct transcriptional mechanisms of the NPY gene in ontogeny. The different profiles of DNA-protein complexes suggest recruitment of distinct mechanisms at different sites on the NPY promoter in DNA binding with nuclear factors. Additionally, in the NZB mouse, a defect in binding of nuclear factors to NPY promoter is detected in nuclear extract from the embryo and newborn brain, indicating altered mechanisms of promoter activity early in ontogeny in autoimmunity in the NZB mouse.

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He 90481: A Competitive Nonpeptidergic Antagonist at Neuropeptide Y Receptors

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INTRODUCTION

Neuropeptide Y (NPY) has a widespread localization in many neurons of the central and peripheral nervous system and exogenous NPY elicits numerous physiological effects.[1] The ultimate assessment of the physiological relevance of NPY, however, will require the use of specific antagonists. Moreover, such antagonists may also be useful in the pharmacological characterization of possible subtypes of NPY receptors which have been proposed but not unequivocally defined. The present study used NPY-stimulated mobilization of intracellular Ca^{++} in human erythroleukemia (HEL) cells[2,3] as a model system to determine the functional effects of He 90481 on NPY receptors.

MATERIALS

He 90481 (FIG. 1) and porcine NPY were kindly provided by Heumann Pharma (Nürnberg, Fed. Rep. Germany) and by Drs. J. E. Rivier and J. H. Boublik (Salk Institute, La Jolla, CA), respectively. Fura-2 was purchased from Molecular Probes (Eugene, OR).

RESULTS AND DISCUSSION

NPY increased the free intracellular Ca^{++} concentration of HEL cells with an EC_{50} of $\approx$ 5 nM (FIG. 2), whereas He 90481 (in concentrations up to 100 μM) had no direct effect (data not shown). However, He 90481 shifted the concentration-response curves for NPY to the right (towards higher concentrations) without altering the maximal NPY-stimulated Ca^{++} increase (FIG. 2). Schild plots were linear with a slope which was not significantly different from unity (1.01 $\pm$ 0.25; n = 3). A pA_2 value of 4.43 (affinity = 37.5μM) was calculated from these data.

These data demonstrate that He 90481 is a competitive antagonist at NPY receptors.

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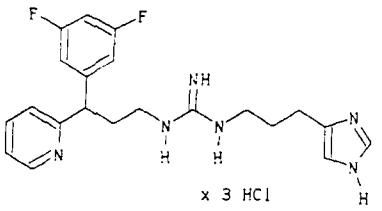

FIGURE 1. Chemical structure of He 90481.

The usefulness of He 90481 as a NPY antagonist, however, is limited by two factors: i) Its affinity at NPY receptors is quite low, and ii) it is not specific for NPY receptors. In the concentration range where He 90481 blocks NPY receptors, it also competes for α_2-adrenergic receptor binding (unpublished observations). Moreover, He 90481 is an agonist at H_2 histamine receptors of guinea pig heart with a potency approximately 1000 times greater than its affinity at NPY receptors (Dr. Engler, Heumann Pharma, personal communication). Further studies with related compounds may yield nonpeptidergic NPY antagonists with higher affinity and greater specificity.

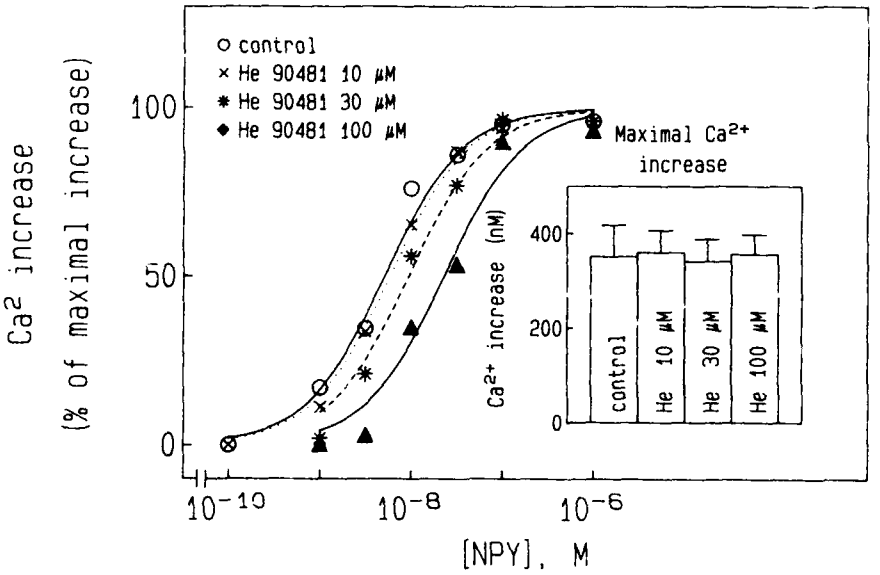

FIGURE 2. Effects of various concentrations of He 90481 on NPY-stimulated Ca^{++} mobilization in HEL cells. HEL cells were grown in RPMI medium and free intracellular Ca^{++} concentration of these cells was determined with the fluorescent indicator dye Fura-2 as recently described.[2] Concentration-response curves for NPY were constructed in the absence and presence of various concentrations of He 90481. The curves were generated by fitting the experimental data to a sigmoid curve; in these calculations the bottom of the curve was fixed at 0 and the Hill-slope at 1. The data were then expressed as % of the maximal response obtained in the experiment, and these percentages were averaged to obtain the mean concentration-response curves shown. The mean absolute increases caused by NPY in the absence and presence of He 90481 are shown in the inset. Data are mean (in the inset mean ± SEM) from three experiments.

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Neuropeptide Y and Peptide YY Interact with Rat Brain Sigma and PCP Binding Sites

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The existence of endogenous ligands "sigmaphins" for sigma opioid receptors was first demonstrated by Quirion *et al.*[1] in pig extracts. This finding has largely been assessed by several authors reporting the existence of peptidergic fractions from guinea pig, pig and human brain exhibiting affinity for sigma or PCP receptors, or both.

The purpose of the present report is to describe the interactions of neuropeptide Y (NPY) and other related neuropeptides with the sigma binding site labeled with (+)3H-SKF10,047 ((+)3H-NANM) or 3H-DTG and with the PCP binding site labeled with 3H-TCP in rat brain membranes.

METHODS

Brain membranes prepared from male Sprague-Dawley rats weighing 200–225 g (IFFA-CREDO, France) were used for binding studies with (+)3H-NANM (40 Ci/mmole),3H-DTG (52.3 Ci/mmole), or 3H-TCP (46 Ci/mmole). The incubations were carried out according to Largent *et al.*[2] in a final volume of 250 µl. All peptides were from Peninsula and ligands were from New England Nuclear Corp. except for 3H-TCP (CEA, FRANCE).

RESULTS AND DISCUSSION (TABLE 1)

1. PYY and NPY displayed an IC50 <10 nM for the displacement of (+)3H-NANM. NPY appeared to be specific for the (+)3H-NANM binding site.
2. PYY was devoid of specificity, and besides its high affinity for the sigma binding sites it was more active than PCP itself on 3H-TCP binding sites.
3. The existence of 2 domains for sigma ligand binding already hypothetized by others[3] is confirmed by our results showing the differentiation between a (+)benzomorphan domain binding the (+)benzomorphans such as (+)NANM and pentazocine and a nonbenzomorphan domain binding nonbenzomorphan sigma ligands such as (+)3PPP and DTG.

CONCLUSIONS

These results suggest that NPY and to a lesser extent PYY could be the endogenous ligand interacting with the (+)benzomorphan binding site. On the other hand, PYY and to a lesser extent NPY, could be the endogenous ligand interacting with the PCP binding site.

TABLE 1. Binding Affinities of NPY and Related Peptides for the Rat Brain Sigma Binding Sites Labeled with Various 3H-Ligands and for PCP Binding Sites Labeled with 3H-TCP[a]

Compounds	(+)3H-NANM		3H-DTG		3H-TCP	
Haloperidol	24.6 ± 6.7	(6)	52 ± 13	(5)	>10000	(3)
(+)NANM	98 ± 17	(4)	4543 ± 1741	(4)	343 ± 57	(4)
DTG	253 ± 57	(3)	108 ± 15	(3)	9024 ± 1604	(3)
R(+)3PPP	789 ± 376	(7)	106 ± 21	(4)	>10000	(3)
(±)Pentazocine	38 ± 5.6	(3)	254 ± 53	(4)	2879 ± 197	(3)
Rimcazole	2649 ± 636	(4)	2503 ± 1033	(3)	>10000	(3)
BMY14802	3216 ± 1122	(3)	271 ± 46	(4)	>10000	(3)
PCP	601 ± 371	(4)	5485 ± 1778	(4)	61 ± 7.7	(3)
NPY	10 ± 5	(4)	−18%[b]	(3)	−7%[b]	(3)
PYY	4.9 ± 0.8	(4)	−39%[b]	(3)	32 ± 4	(2)
PP	−42%[b]	(4)	−37%[b]	(3)	−14%[b]	(3)
Neurotensin	0%[b]	(4)	0%[b]	(3)	0%[b]	(3)
Bombesin	−17%[b]	(3)	−11%[b]	(3)	−16%[b]	(4)
Somatostatin	−23%[b]	(3)	−12%[b]	(4)	−20%[b]	(3)
Substance P	−25%[b]	(4)	−5%[b]	(3)	−14%[b]	(3)
VIP	−47%[b]	(3)	−10%[b]	(3)	−9%[b]	(3)
TRH	−8%[b]	(3)	0%[b]	(3)	0%[b]	(3)

[a]Values are the mean IC50 (nM) ± SEM from the number of independent experiments indicated in brackets.

[b]In the case of peptides, when 50% inhibition was not reached at 100 nM, the activity was given in terms of percent inhibition at that concentration.

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Characterization and Use of Four Anti-NPY Monoclonal Antibodies to Study NPY-Receptor Interaction

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Neuropeptide Y (NPY) is a 36-amino acid peptide present abundantly in sympathetic perivascular nerves and different areas of the brain. Several lines of evidence suggest that NPY is involved in cardiovascular regulation.[1,2] We describe the production and characterization of four anti-NPY monoclonal antibodies (NPY02, NPY03, NPY04 and NPY05) and their use to study the NPY-receptor interaction.

Total NPY and NPY 27–36 were conjugated to keyhole limpet hemocyanin (KLH, Sigma) with glutaraldehyde as the coupling reagent.

Immunizations, cell fusion, hybridoma selection, cloning and ascite production were performed according to standard procedures.[3] The epitopes of these antibodies were mapped using 25 different fragments and related peptides of NPY. The characteristics of the antibodies are given in TABLE 1.

The interactions between NPY and the different antibodies were investigated using a preparation of rat brain membranes known to contain a lot of binding sites of NPY using standard methods.[4]

The radioreceptor assay was performed by incubating 100 μg of these membranes with iodinated NPY (total binding). Nonspecific binding was obtained in the presence of 1 μM of cold NPY. Specific binding was about 70% of the total binding.

The 4 monoclonal antibodies were preincubated with iodinated NPY for 2 hours at room temperature. The mixture was then added to the rat brain membrane preparation for 90 minutes and the binding of the monoclonal antibody–NPY complex to the receptor measured. FIGURE 1 shows the influence of the different antibodies on the binding of radiolabeled NPY on the membranes. NPY02 or NPY05 abolished binding of NPY to its receptor. FAb of NPY05 were obtained by papain digestion. These fragments were still able to bind to NPY. NPY03 had no effect on NPY-receptor interaction but, unexpectedly, NPY04 produced a consistent enhancement of NPY-receptor binding. Iodinated NPY04 was found not to bind to the membranes in the absence of NPY (data not shown), suggesting that the binding of the complex ^{125}I NPY–NPY04 is not due to a Fc receptor.

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TABLE 1. Characteristics of NPY Antibodies

Antibody	Epitope	K_a	Isotype
NPY02	NPY 11–24	5.5×10^{10}	IgG1
NPY03	NPY 27–33	6.7×10^{9}	IgG2
NPY04	NPY 01–12	3.8×10^{8}	IgG3
NPY05	NPY 32–36	2.5×10^{10}	IgG2

Moreover, the binding of iodinated NPY04 to the membranes was dependent on the concentration of cold NPY added to the medium.

Thus, the availability of monoclonal antibodies directed against precisely defined epitopes of NPY represents a useful tool not only for developing sensitive and specific two-site immunoradiometric assays of NPY,[5] but also for the study of structure-function relationships and, possibly, the cloning of the NPY receptor gene.

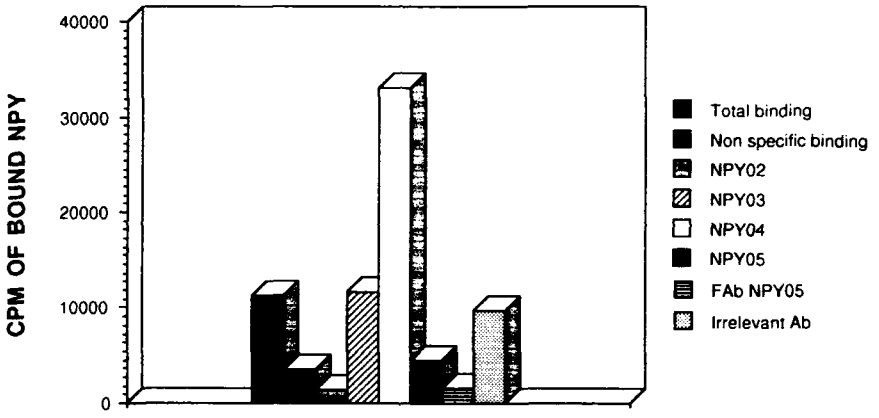

FIGURE 1. Effect of 4 anti-NPY monoclonal antibodies on binding of NPY to rat brain membranes. The binding of iodinated NPY is expressed as CPM.

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Neuropeptide Y and Its C Terminal Fragments Attenuate Anion Secretion in Rat Jejunum Mucosa[a]

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Neuropeptide Y (NPY) is present within intrinsic nerves innervating gastrointestinal smooth muscle and mucosa in the rat.[1] Submucous neurones predominantly innervate the mucosal area and those that contain NPY-like immunoreactivity form particularly dense networks of fibres in the villus area of the rat small intestine. This dense innervation is indicative of a functional role for NPY and indeed this peptide is a potent antisecretory agent,[2] as is Peptide YY (PYY), reducing basal[2] as well as secretagogue-evoked chloride secretion[3] in rat jejunum. These effects are mediated by direct activation of basolateral epithelial receptors specific for NPY/PYY.[4] The aim of this study was to establish the type of NPY receptor (Y_1 or Y_2-like) involved in these antisecretory actions by comparing the effects of a range of C terminal fragments of NPY with those of the full length neuropeptide.

Overlying smooth muscle was removed by blunt dissection and the remaining mucosal sheets were placed between two halves of an Ussing chamber and voltage clamped as described previously.[2] Tissues were bathed in Krebs-Henseleit buffer and gassed with 95% O_2/5% CO_2. All preparations were allowed to attain a steady basal short circuit current (scc) before initiating investigations. In certain studies where electric field stimulation (EFS) was performed, a pair of Ag sheet electrodes was placed either side of the tissue in order to deliver rectangular pulses (of 0.6 msec duration and 5 Hz frequency, for 1 second). These trains of stimuli were delivered at 7-min intervals while continuously recording changes in scc. These stimulation parameters elicit rapid transient increases in scc that are repeatable and remain unchanged in the absence of any blockers. When comparing the effects of NPY and fragments upon secretory responses generated by EFS, values were quoted as % of controls in the presence of hexamethonium and atropine.

Full length NPY reduced basal scc with an EC_{50} of 10 nM (FIG. 1). The C terminal fragments (11–36), (12–36), (13–36) and (14–36) NPY appeared to be full agonists though much less potent than NPY (EC_{50} of approximately 1 μM). Shorter fragments also attenuated basal scc but these were less potent still. A similar order of potency was obtained when assessing the attenuation of EFS-generated secretion by NPY and fragments. NPY attenuated EFS secretory response in a concentration-dependent manner (EC_{50} of 3 nM, data not shown), 30 nM NPY virtually abolishing EFS responses 14–28 min after application (FIG. 2). This was compared with the lesser effects of 300 nM concentrations of each fragment. As the peptides became shorter their inhibition was less marked and of shorter duration. (26–36) NPY, desamido NPY and the C terminal flank-

[a]Supported by a project grant from the Medical Research Council, UK.

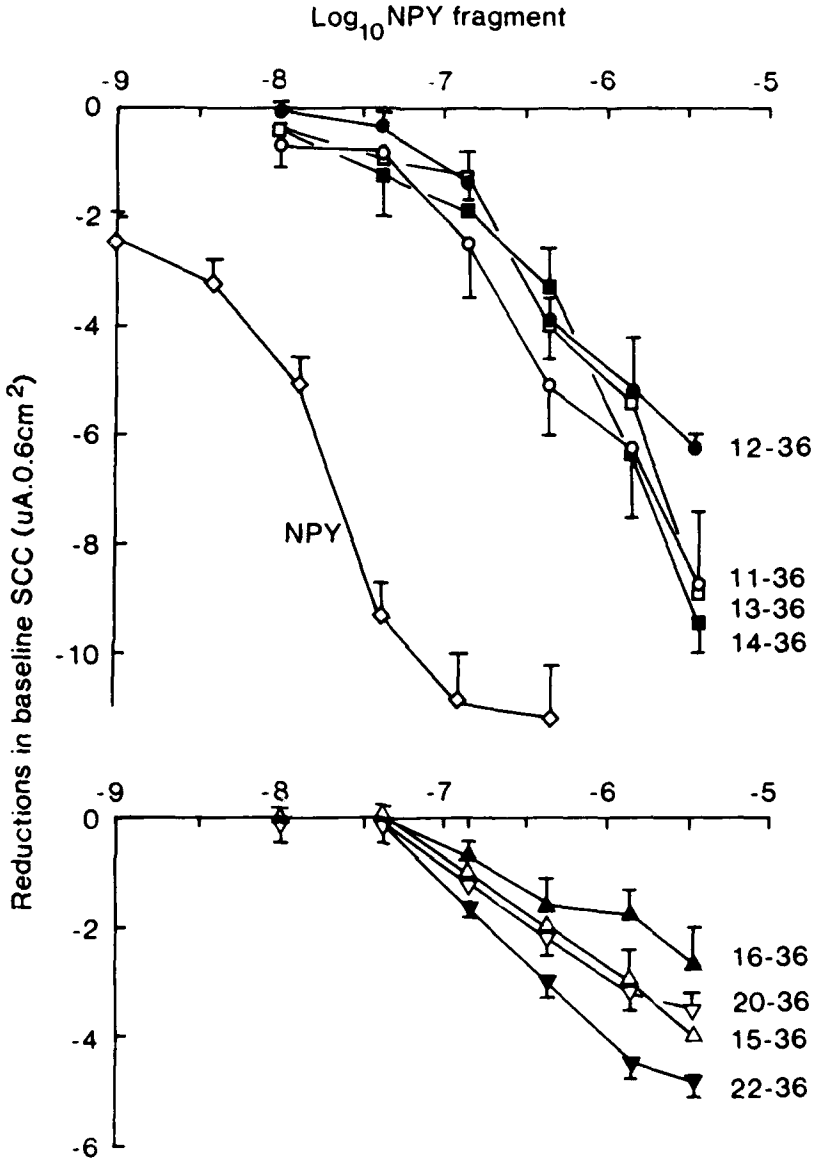

FIGURE 1. Antisecretory effects of NPY and fragments upon baseline scc. All peptide additions were made to the basolateral surface in a cumulative fashion and reductions in scc were recorded as $\mu A.0.6cm^{-2}$. Each point represents the mean with 1 SEM (some errors are omitted for reasons of clarity). The number of observations for each fragment are as follows: NPY, 3–11; NPY (11–36) 3–9; NPY (12–36) 2–9; NPY (13–36) 2–11; NPY (14–36) 2–9; NPY (15–36) 2–7; NPY (16–36) 2–7; NPY (20–36) 3–7; NPY (22–36) 3–7.

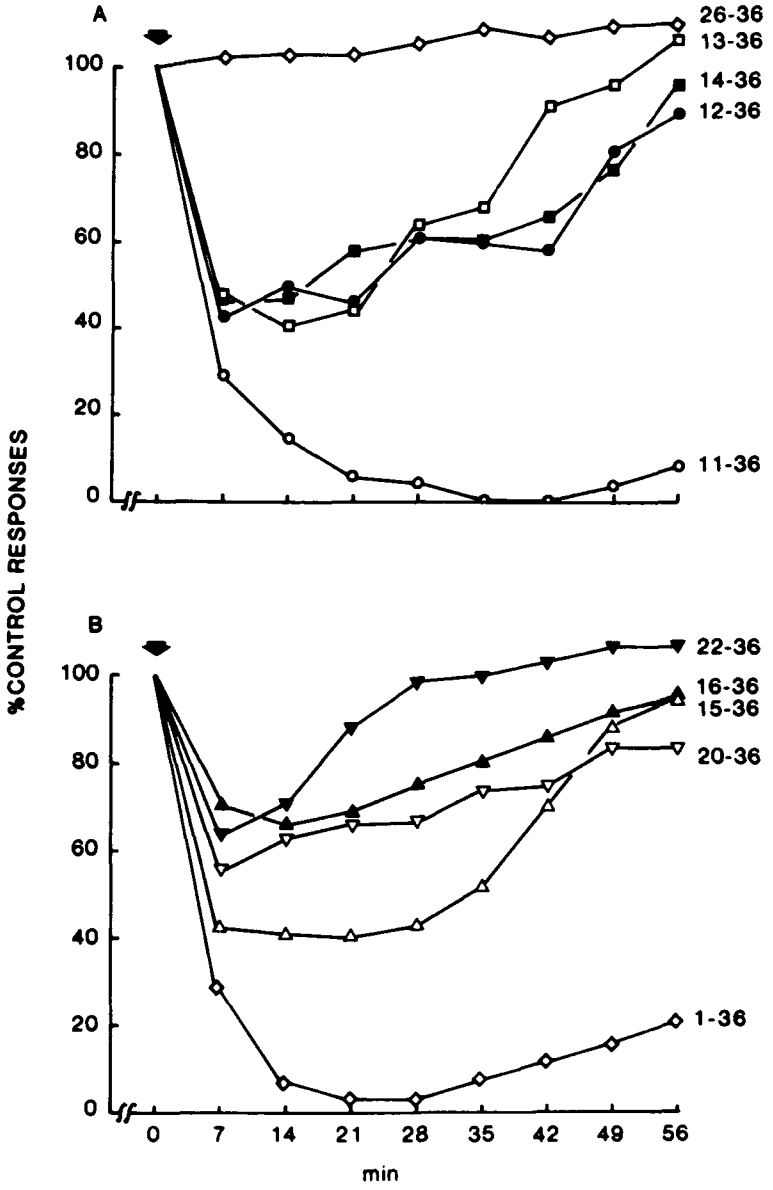

FIGURE 2. The effect of a range of NPY fragments upon hexamethonium/atropine-resistant EFS responses. Control secretory responses were obtained in each tissue in the presence of hexamethonium and atropine before application of NPY fragments (at 300 nM throughout) or 30 nM NPY (1–36). The figure is split into **A** and **B** for reasons of clarity. Control EFS responses were denoted as 100% and all subsequent increases in scc after EFS were calcuiated as a percentage of each tissue control. Control EFS responses as $\mu A.0.6cm^{-2}$ were as follows with n values in parenthesis: 6.1 ± 0.5 (4) for NPY (1–36); 2.9 ± 0.4 (4) for NPY (11–36); 1.7 ± 0.2 (3) for NPY (12–36); 1.8 ± 0.2 (4) for NPY (13–36); 3.4 ± 0.8 (4) for NPY (14–36); 5.5 ± 0.8 (4) for NPY (15–36); 6.5 ± 0.5 (4) for NPY (16–36); 4.7 ± 0.3 (4) for NPY (20–36); 6.9 ± 1.7 (4) for NPY (22–36) and 6.6 ± 0.8 (4) for NPY (26–36). All points and values are quoted as means and the errors are not shown in the figure for reasons of clarity, but were not greater than 10%.

ing peptide of NPY (CPON) were inactive, both at reducing basal scc and EFS secretion, and they had no effect upon NPY responses per se.

In conclusion the antisecretory effects observed with NPY and its C terminal fragments indicate that the receptors mediating these responses in the rat jejunum are Y_2-like.

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Neuropeptide Y Gene Expression Is Dependent upon a SP1-Like DNA-Binding Protein

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Neuropeptide Y (NPY) is the most abundant and widely distributed neuropeptide in the mammalian nervous system. Elucidating the molecular mechanisms responsible for its pattern of expression will provide insight into how the diversity of gene expression in the nervous system is manifested.

A series of expression plasmids were constructed which contain varying amounts of the NPY gene's 5'-flanking sequences fused to the coding sequences of the bacterial enzyme, chloramphenicol acetyltransferase (CAT). These deletion constructs were transiently introduced into the human Lan-5 cell line, which produces authentic NPY. These experiments demonstrated that the sequences between -63 and -51 were necessary for expression of this gene. Gel retardation analyses of this region indicated that a SP1-like molecule bound to these sequences. A Southwestern analysis was performed in which Lan-5 cell and HeLa cell nuclear extracts were probed with the NPY promoter sequences (-63 to -51) or the SV40 promoter (GC boxes IV and V). In each case, a band of approximately 97 Kd was apparent. This was in good agreement with purified SP1.

An in vitro transcription system was created from Lan-5 cell nuclei which accurately transcribed the NPY/CAT fusion constructs. The data obtained from these experiments also demonstrated that the sequences between -63 and -51 were necessary for transcription. Competition experiments in which excess oligonucleotides containing SP1 consensus sequences were added to the transcription reactions reduced transcription from the NPY promoter by 80%.

Maximal expression of the NPY gene in transient transfection and in vitro transcription experiments was obtained when sequences up to -246 were included in the promoter construct. This region of the NPY promoter contains several consensus sequences for ubiquitous DNA-binding proteins such as SP1, AP1, and CAAT. DNAse I protection analysis of the region between -118 and +1 indicated that extensive DNA/protein interactions were possible. The functional significance of these interactions is currently unknown.

Addition of sequences up to -1078 resulted in decreased expression of the NPY gene both in the in vivo transfection and in vitro transcription experiments. In contrast to the proximal 5'-flanking sequences, analysis of the region between -1078 and -796 showed that it was 67% AT nucleotides and contained the potential for forming bent DNA. The role these sequences play in NPY gene expression is currently under investigation.

Sympathetic Stimulation-Evoked Overflow of Neuropeptide Y and Norepinephrine from the Heart

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Neuropeptide Y (NPY) and norepinephrine are stored in and released from cardiac sympathetic nerve terminals. Although previous studies have shown that norepinephrine (NE) overflow returns rapidly to control after the cessation of sympathetic stimulation, much less is known about the washout of neuropeptide Y from the heart. In 9 anesthetized dogs we measured the overflow of neuropeptide Y and norepinephrine evoked by 3-minute trains of sympathetic stimulation applied at frequencies of 5 Hz and 10 Hz. We collected blood samples from the coronary sinus and descending aorta before, during, and after sympathetic stimulation. The plasma content of neuropeptide Y was determined by radioimmunoassay and that of norepinephrine by HPLC with electrochemical detection. To calculate the overflow we multiplied the venous-arterial concentration difference by the coronary sinus blood flow. Sympathetic stimulation increased the overflow of norepinephrine and neuropeptide Y from the heart in a frequency-dependent manner ($p < 0.001$) (TABLE 1).

After the cessation of the 5 and 10 Hz trains of sympathetic stimulation, the norepinephrine overflow returned to basal levels within 5 minutes; however, the neuropeptide Y overflow returned to basal levels only after 20 and 60 minutes, respectively. Although the much slower decay rate of neuropeptide Y overflow may be explained by differences in the removal mechanisms (*e.g.*, diffusion) for these two molecules from the cardiac neuroeffector junctions, the substantial disparity in the decay rates indicates that differences in other neuroeffector processes (*e.g.*, agonist-receptor interactions) may also play a role.

TABLE 1. Overflow of NE and NPY from the Heart during Sympathetic Stimulation

	5 Hz	10 Hz
NE (ng/min)	928.4 ± 135.7	5538.6 ± 1171.3
NPY (ng/min)	9.4 ± 3.1	47.7 ± 17.2

Effect of Neuropeptide Y on the Biphasic Response of the Rabbit Ear Artery to Norepinephrine and Sympathetic Field Stimulation

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In addition to its ability to constrict blood vessels, neuropeptide Y (NPY) can potentiate the response to other constrictor agents and to sympathetic field stimulation. We showed previously that NPY will potentiate the response of the isolated perfused rabbit ear artery to brief trains of field stimulation (300–500 msec).[1,2] To further characterize the facilitatory effect of NPY on sympathetic neurotransmission, we studied its effect on extended stimulation of the perfused ear artery by either sympathetic field stimulation or exogenous norepinephrine (NE). Extended stimulation of this preparation produces a constrictor response which is often separable into two distinct components, an initial phasic response, followed by a tonic response which is maintained for the duration of stimulation.[3]

NPY produced a concentration-related potentiation of both phases of the constrictor response of the ear artery to field stimulation (FIG. 1). In contrast to field stimulation, the initial phasic response could often not be identified in the response to exogenous NE. When the NE-induced constrictor response was expressed as the peak tonic contraction observed during the 2-min drug perfusion, a small and highly variable potentiation by NPY was observed (20 ± 10%, n = 16). However, as observed in the rat caudal artery,[4] the rate of onset of the NE-induced contraction appeared to be enhanced. This resulted in a selective potentiation of the phasic response in those arteries where the two phases could be clearly discerned.

To quantitate this effect, an additional series of experiments was performed, using a differentiator to calculate the maximum slope of the contractile response. In this manner, NPY was shown to produce a statistically significant potentiation of the peak rate of the NE-induced contraction. However, in contrast to the contractions induced by field stimulation, NPY did not produce a significant potentiation of the tonic response to exogenous NE (FIG. 2), although there were individual experiments where NPY produced an equivalent potentiation of both phases of the response.

These results show a difference in the effect of NPY on the response of the perfused rabbit ear artery to exogenous vis-à-vis endogenous NE. A selective potentiation of the phasic response was observed only in the case of exogenous NE. The phasic and tonic

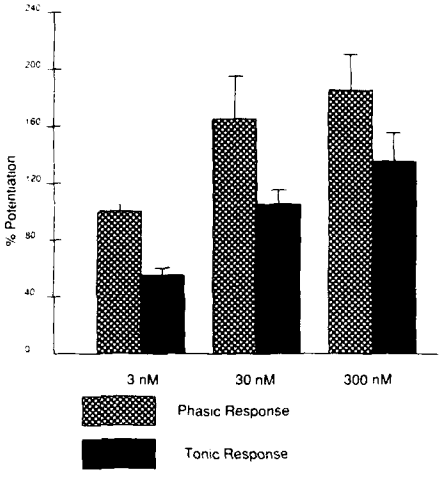

FIGURE 1. Effect of intraluminal administration of NPY at 3, 30 and 300 nM on the phasic and tonic vasoconstrictor responses elicited by field stimulation of the isolated perfused rabbit ear artery (2 Hz, 80 V, 0.7 msec pulse duration) applied for 2 min at 10-min intervals. Each bar represents the mean of 5 experiments ± SEM. Since the response to field stimulation was clearly biphasic in all cases, the magnitude of the phasic and tonic responses could be measured directly from the polygraph tracing.

responses in the ear artery are thought to be mediated by intracellular calcium release and extracellular calcium translocation, respectively.[3] Calcium translocation is apparently involved in the potentiation by NPY of the response to field stimulation in the ear artery, based on attenuation of the NPY effect by nifedipine.[2] Hence the differential action on the responses exogenous and endogenous NE may suggest differences in the involvement of the two calcium pools. Alternatively, the differences may relate to the anatomical site of action, with exogenous NE directed toward the intimal smooth muscle but the endogenous neurotransmitter released at the medial-adventitial junction.

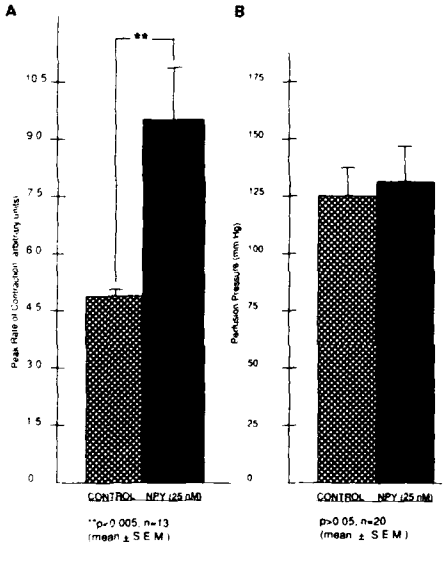

FIGURE 2. Effect of intraluminal administration of NPY on the vasoconstrictor response elicited by intraluminal norepinephrine infusion to the isolated perfused rabbit ear artery (100 nM for 2 min at 10-min intervals). Peak rate of contraction **(A)** was determined by electronic differentiation of the pressure response. The tonic response **(B)** was measured as the maximum perfusion pressure attained during the norepinephrine infusion. This summary data includes some experiments where only the tonic response was measured.

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Platelets as a Source and Site of Action for Neuropeptide Y[a]

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Despite the conventional view that circulating neuropeptide Y (NPY) reflects neuronal release, platelets are the largest source of circulating immunoreactive-NPY (i-NPY) in the rat. Our discovery of i-NPY in rat platelets[1] was based on the observation of NPY mRNA in megakaryocytes of rats and some strains of mice.[2] We observed tenfold greater i-NPY in platelet-rich plasma (PRP) compared to platelet-poor plasma (PPP); i-NPY content was similarly high in platelet pellets prepared from platelet-rich plasma.[1] This i-NPY is released during the secondary, irreversible stage of platelet aggregation and the associated release reaction, but not during the primary, reversible stage. Thus, we speculated that i-NPY from platelets is an important component of the platelet-vascular interaction, in which substances released by platelets and vascular endothelium participate in the complex co-regulation of vascular tone and platelet function.Studies were undertaken to determine whether 1) i-NPY from platelets is the same as neuronal NPY, 2) rat platelets contain binding sites for NPY, and 3) NPY affects platelet function.

To examine the chemical character of i-NPY, PRP was prepared from anticoagulated rat blood and was stimulated maximally with collagen to induce secondary aggregation. After aggregation and rapid centrifugation, the supernatant was extracted with ethanol, filtered, and subjected to reverse phase HPLC using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected and assayed for i-NPY. Porcine NPY standards and PPP were also run under identical conditions. The elution profile of porcine NPY standard consisted of a single immunoreactive peak (FIG. 1a), whereas a complex profile was obtained for PPP (FIG. 1 b), as generally reported for rat plasma. Of the two major peaks, the second had a retention time slightly less than porcine NPY, as expected for rat NPY. Supernatant from stimulated PRP also had two major peaks, one in the position expected for rat NPY. Notably, this peak was 11-fold higher in PRP compared to PPP; the earlier peak was elevated 4-fold. These data suggest that rat platelet i-NPY is

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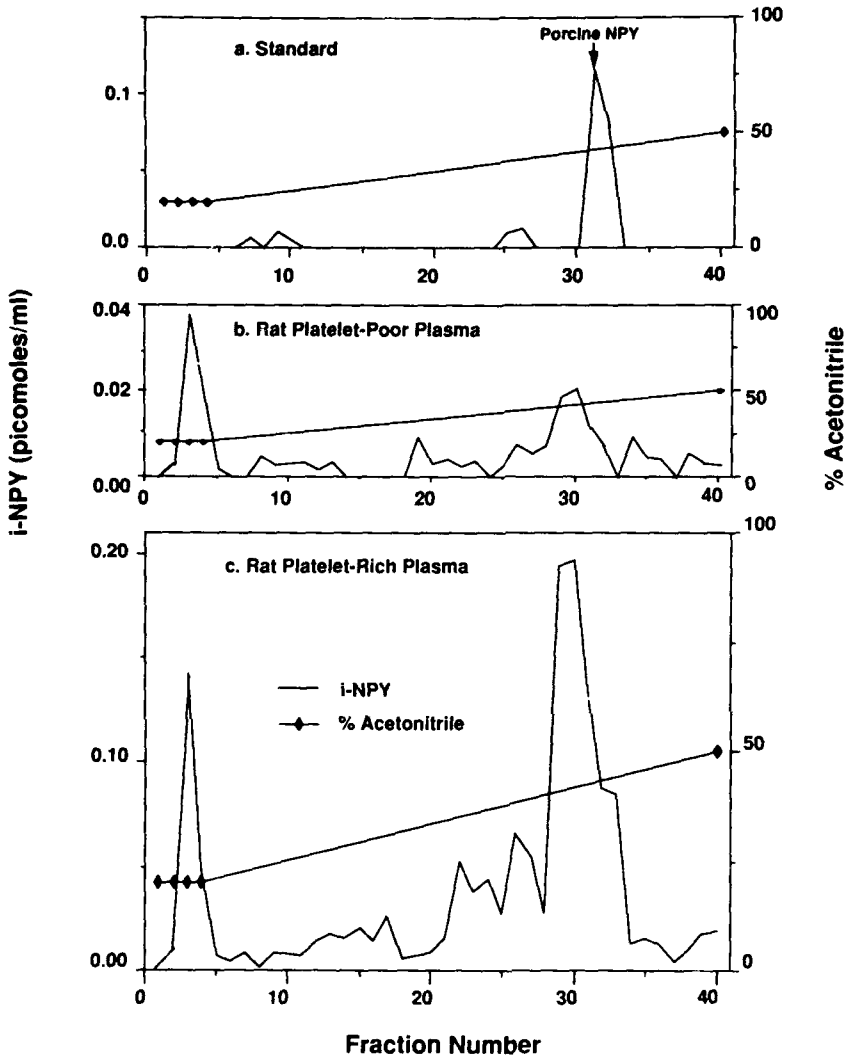

FIGURE 1. Reverse phase HPLC profile of i-NPY in rat plasma. Porcine NPY standard yielded one immunoreactive peak (**a**), whereas two major peaks of i-NPY were observed in platelet-poor plasma (**b**) and the supernatant from platelet-rich plasma maximally aggregated by addition of collagen (**c**). The second major peak in both cases had a retention time slightly less than the porcine NPY standard, suggestive of its identity as authentic rat NPY. This peak was 11-fold higher in the supernatant of stimulated platelet-rich plasma than in platelet-poor plasma, demonstrating its release during aggregation.

authentic NPY. Ogawa *et al.*[3] recently reported that several different HPLC methods yield similar results, with coelution of rat platelet-derived i-NPY and authentic rat NPY. Interestingly, Ogawa *et al.* observed substantially more platelet i-NPY in spontaneously hypertensive rats compared to normotensive rats.

To investigate binding of NPY to rat platelets, platelets isolated from rat PRP were homogenized, and membranes were prepared by ultracentrifugation and washing with 5 mM Tris-HCl, with the final resuspension of membranes in 25 mM Tris-HCl (pH 7.4). Specific and nonspecific binding of ^{125}I-NPY was measured by standard methods after 60 min incubation at 22°C. Scatchard analysis revealed specific, saturable binding sites for NPY in rat platelets, with a Kd of approximately 0.8 nM (similar to affinity in brain and iris); the Bmax was approximately 470 fmol/mg protein. Preliminary experiments demonstrate displacement of binding by NPY 13–36, suggesting a Y2-type binding site.

We previously reported that NPY has no direct stimulatory or inhibitory effects on aggregation of rat platelets, but that under some conditions, there is a tendency toward

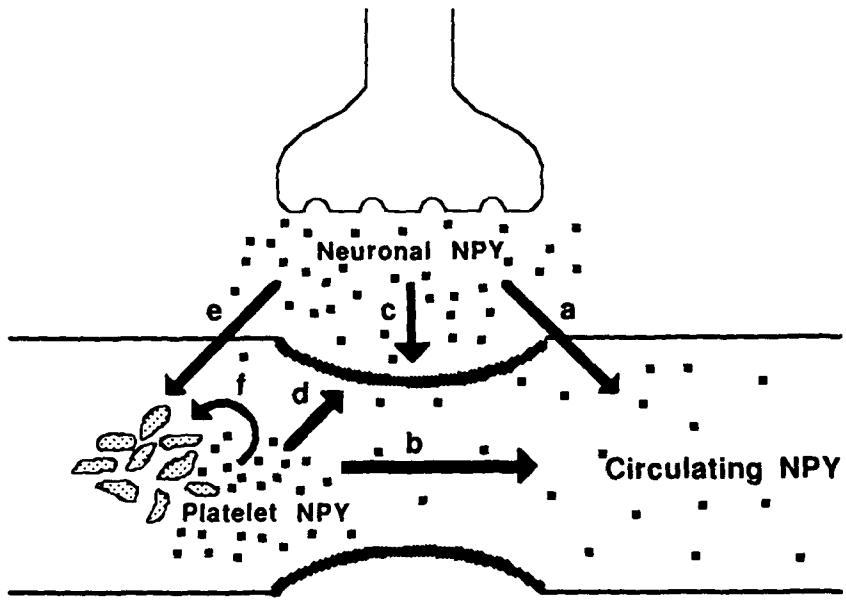

FIGURE 2. Proposed role of NPY in the platelet-vascular interaction. Circulating i-NPY in the rat represents both neuronal (a) and platelet (b) derived NPY. NPY from both sources contributes to vasoconstriction during cardiovascular states involving both sympathetic nervous system and platelet activation (c and d), for example during severe stress, or shock. During thrombosis, platelet-derived NPY (d), along with other vasoactive platelet products, promotes vasoconstriction. Both neuronal and platelet NPY bind to and might also affect function of platelets (e and f).

potentiation of aggregation by other agonists.[1] Recently, it was reported that NPY inhibits epinephrine-induced aggregation of human platelets.[4] We have repeated those experiments and are unable to confirm this effect under our laboratory conditions in our population of subjects. Briefly, PRP was prepared from anticoagulated blood from volunteers, and aggregation was measured in a transmittance aggregometer, using sufficient epinephrine to stimulate secondary aggregation. Only aspirin-free volunteers displaying both primary and secondary aggregation in response to epinephrine were included in the study. Aggregation was tested in the presence of human or porcine NPY, 10^{-9} to 10^{-5} M, or vehicle. No inhibition of aggregation by NPY was observed in human PRP, with the same results in gel-filtered human platelets. In contrast, in rat platelets, we continue

to observe a tendency toward potentiation of aggregation by threshold doses of platelet agonists such as collagen; this is an area of active investigation in this laboratory.

Based on our studies, we propose that NPY is an important component of the platelet-vascular interaction in species in which platelets contain and release NPY (FIG. 2). Such a role has been defined for other vasoactive substances released by platelets, including serotonin, thromboxane, platelet activating factor, and platelet-derived growth factor. In addition, many platelet release products stimulate or potentiate aggregation. Clearly, circulating i-NPY in some species reflects both platelet-derived i-NPY and NPY from neuronal sources. Release of i-NPY by platelets during pathophysiological states involving platelet activation (*e.g.*, severe stress, thrombosis and shock) should contribute to vasoconstriction, along with NPY released from sympathetic nerve endings. Furthermore, because platelets in the rat, and possibly other species, contain binding sites for NPY, platelets represent a potential site of action for NPY released through either sympathetic or platelet activation. The nature of this action is under investigation.

Along with the effect of NPY on platelet function, several important questions remain: 1) Do platelets from other species contain and release i-NPY? 2) Are binding sites for NPY universal in mammalian platelets? 3) Are platelet levels of i-NPY altered in disease states such as hypertension? The answers to such questions should yield important insight into the role of NPY in the platelet-vascular interaction and in broader areas of cardiovascular pathophysiology.

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Sources and Vasopressor Efficacy of Circulating Neuropeptide Y during Acute and Chronic Stress in Rats[a]

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Previously we reported that rat platelets contain large amounts of neuropeptide Y immunoreactivity (NPY-ir), releasable during aggregation.[1] Thus, in rats, platelets as well as the sympatho-adrenomedullary system are potential sources of circulating NPY. Conceivably, both sources may contribute to the increased plasma NPY-ir levels observed in rats during certain types of stress.[2] The vasoactivity of sympatho-adrenomedullary and/or platelet-derived NPY in stress is unknown. However, NPY is a vasoconstrictor whose efficacy is markedly enhanced by norepinephrine;[3] and since stress elevates circulating catecholamines,[4] we hypothesize that the role of NPY in stress-induced vasoconstriction may also increase. In the present study we evaluated which sources, neuronal, extraneuronal, or both, contribute to circulating NPY levels at rest, and during acute and chronic stress, using sympathetic ganglionic blockade or heparin (at the dose which inhibits platelet aggregation).

The studies were performed on male albino rats (300–400 g), cannulated for blood pressure measurement, blood sampling (femoral artery), and drug injections (femoral vein) 48 hours before the experiments. Two stress paradigms were used. In an acute stress of cold water exposure (COLD), rats were placed for 10 min in plastic cages with 1 cm deep ice-cold (4°C) water. In the footshock stress (FS), rats were placed in shock chambers where electrical footshock was delivered through the floor grid at 1.5 mA, 0.5 msec duration for 5 min. In chronically stressed rats, FS was delivered daily for 30 days. Blood samples for NPY-ir were collected before, immediately after, and 20 min after stress. Plasma NPY-ir levels were measured in acid-ethanol extracted plasmas by radioimmunoassay using NPY antiserum (Peninsula Lab.), ^{125}I-NPY (Amersham), and porcine NPY as standard, as previously described.[4]

Circulating NPY-ir levels increased with both high intensity stressors.[4] Acutely, a ganglionic blocker, chlorisondamine (3 mg/kg, i.p.) prevented COLD- (FIG. 1) induced increments in plasma NPY-ir, as previously shown for norepinephrine,[5] indicating a sympatho-neural origin of these responses. However, COLD-evoked plasma NPY-ir increases were also prevented by an antiaggregatory dose of heparin (1000 units/kg, i.v.).

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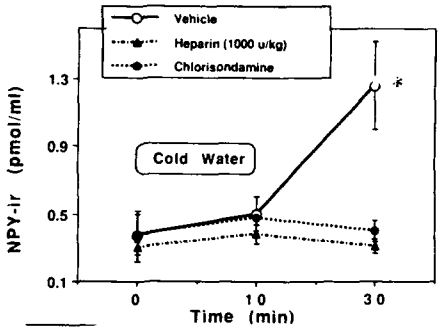

FIGURE 1. Plasma NPY-ir responses to an acute stress of cold (4°C) water exposure in vehicle-, heparin (1000 units/kg, i.v.)-, and chlorisondamine (3 mg/kg, i.v.)-treated rats (n = 4 each group). *p <0.05 as compared to basal, by paired Student t test.

This suggests that platelet release of NPY may occur secondarily to adrenergic activation (which facilitates platelet aggregation), and platelet-derived NPY normally contributes to circulating NPY levels. Chronic chlorisondamine treatment (3 mg/kg, i.p. for 30 days) increased resting plasma NPY-ir levels but prevented the stress-induced increase of NPY (FIG. 2). This may indicate that, chronically, under ganglionic blockade, resting circulating NPY-ir levels increase as a result of the enhanced release from other sources such as platelets. Thus, sympatho-adrenomedullary blockade acutely prevents, but chronically stimulates, the release of NPY from extraneuronal sources.

Chronic FS stress also elevated resting plasma NPY-ir levels but mostly from the sympatho-adrenomedullary system (blocked by chlorisondamine) (FIG. 2). This sympatho-neural pool of NPY, however, was insensitive to stress, since chlorisondamine (given acutely before the last stress) did not prevent increases of plasma NPY-ir in

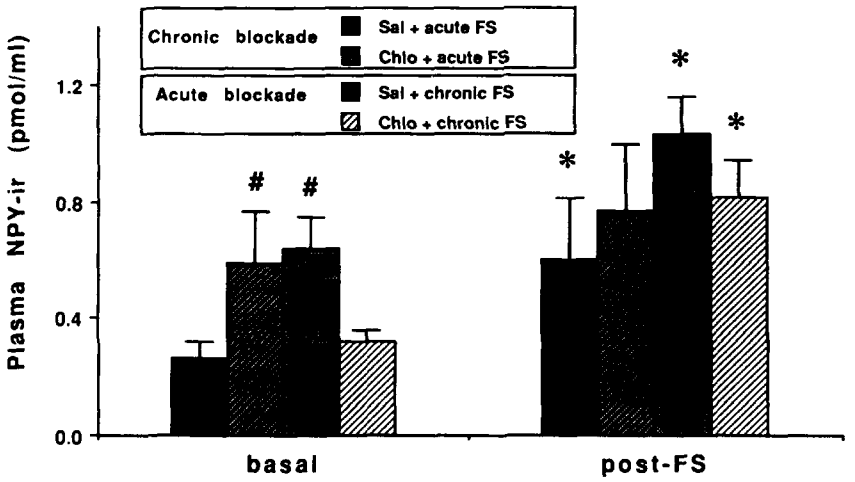

FIGURE 2. Plasma NPY-ir responses to acute or chronic footshock (1.5 mA, 10 min) in rats pretreated acutely or chronically with ganglionic blocker, chlorisondamine (Chlo), or saline (Sal) (n = 5–8). *p <0.05 as compared to basal, and # p <0.05 as compared to chronic Sal + acute FS, by ANOVA and Dunnett t test. Chronic chlorisondamine treatment increased resting NPY-ir levels but prevented plasma NPY-ir response to stress. Chlorisondamine, given acutely, normalized plasma NPY-ir levels elevated by chronic stress.

chronically stressed rats (FIG. 2). Thus, conditions of chronic stress raise resting circulating levels of NPY-ir by stimulating the sympatho-adrenomedullary system, but also sensitize the extraneuronal NPY pool to respond to stress. In contrast to plasma catecholamine responses in chronically stressed stress which become attenuated with repeated stress (a homotypic habituation),[5] plasma NPY-ir levels do not appear to habituate, but rather increase in response to chronic stress. We postulate that this sensitization of NPY responses to chronic stress may be due to multiple sources of circulating NPY which interact with each other in a positive fashion.

The vasopressor efficacy of neuronal and platelet-derived NPY may increase in stress consistently with the hyperresponsiveness to NPY previously found in vivo and in vitro under exposure to high catecholamine concentrations. In support of this notion, NPY caused markedly greater increases in mean arterial pressure in COLD-stressed rats (61.2 + 4.2 mm Hg, n = 6) than in nonstressed rats (30.7 + 3.7 mm Hg, n = 6, $p < 0.01$, at an ED_{30} dose of NPY). Thus, during acute and chronic stress NPY appears to be released into the circulation from both neuronal and platelet sources, becomes a more efficacious vasopressor agent, and may become a predominant mediator of chronic responses to stress when the adrenergic system undergoes habituation (desensitization).

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High Doses of Neuropeptide Y Reduce Blood Pressure in Anesthetized Rats

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Neuropeptide Y (NPY) is a 36-amino acid peptide that is widely distributed in the peripheral nervous system and it is found in many sympathetic fibres.[1] It has been attributed with a variety of actions at the sympathetic neuroeffector junction. NPY causes vasoconstriction and enhances noradrenline-evoked vasoconstriction, thus accounting for the observation that i.v. injection of NPY causes systemic hypertension.[2] Our aim was to further examine the effects of NPY on blood pressure and heart rate (HR) in anesthetized rats.

Male Sprague-Dawley rats were anesthetized with i.p. administration of ketamine and xylazine. The jugular vein and the femoral artery were cannulated with catheters for i.v. administration of the agents and for continuous recording of systemic blood pressure via a pressure transducer connected to a polygraph. In a series of experiments, the histamine H_1-receptor antagonist, mepyramine (10 mg/kg) or the histamine liberator, compound 48/80 (0.12 mg/kg) was given 10 min before i.v. injection of NPY (10 nmol/kg). The preparation for pithing rats is described elsewhere.[3]

NPY raised the mean arterial blood pressure (MAP) dose-dependently (FIG. 1). The pressor response was maximal 20 s after the injection, lasted for about 30 s and was followed by a dose-dependent fall in blood pressue at doses of 3.0 nmol/kg and higher. Maximum hypotension was observed 1 min after injection of 10 nmol/kg NPY. At the same time the HR was 300 ± 32.2 compared to a starting value of 260 ± 15.2, $p > 0.05$ ($n = 5$). After repeated administration of NPY (10 nmol/kg with 10-min intervals), the amplitude to the pressor response was not affected but the depressor response disappeared. Pretreatment with mepyramine or compound 48/80 did not change the amplitude of the pressor response to NPY; the depressor response, however, disappeared (FIG. 2). The blood pressure remained elevated several min after the NPY injection. Pithed rats were given 30 nmol/kg NPY i.v. NPY initially evoked a pressor response; the MAP increased by 134 ± 5% from a baseline of 64 ± 2 mmHg, with a maximum approximately 1 min after the injection. There was a slight increase in HR 1 min after injection of NPY, +4.5 ± 0.8 %, $p < 0.01$ ($n = 4$). The pressor response was followed by a depressor

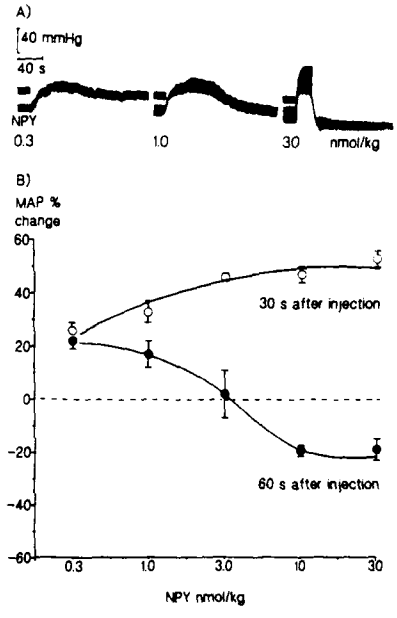

FIGURE 1. **(A)** Recordings showing arterial blood pressure responses to increasing doses of NPY (each dose was given to a naive rat). Injection of NPY is indicated. **(B)** Dose-response curves show the % change of the mean blood pressure (MAP) in response to NPY after 30 s (○) and after 60 s (●); means ± SEM, n = 4–8.

response which reached a maximum 7–12 min after the NPY injection. The depressor response was 20 ± 3% decrease with respect to the starting value.

In this study we show that, after injection of high doses of NPY, the pressor response was followed by a dose-dependent fall in blood pressure in intact as well as in pithed rats. When high doses were given repeatedly, the amplitude of the pressor response was not affected whereas the depressor response disappeared. The depressor response was abolished by pretreatment with mepyramine or compound 48/80, suggesting that NPY lowers blood pressure by releasing histamine from a mast cell pool.

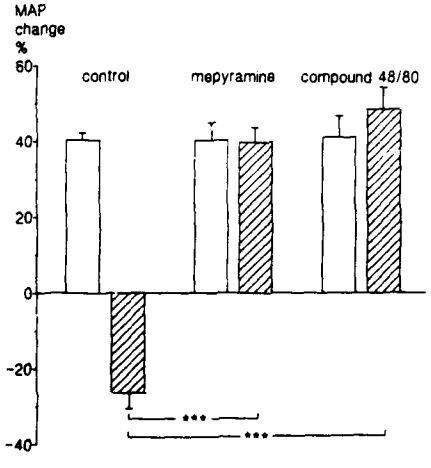

FIGURE 2. Bars showing pressor response in % of starting level of mean arterial blood pressure (MAP) to NPY (10 nmol/kg) after 20–30 s *(white)* and depressor (or lack of depressor) response after 60 s *(shaded)*. Means ± SEM n = 4–5, $p < 0.001$.

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Neuropeptide Y (NPY), NPY-Immune Complex, and NPY(18–36) Effects on Cardiovascular Function[a]

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Neuropeptide Y (NPY) at low doses (0.2–0.6 nmoles) given intra-arterially (ia) increased systemic vascular resistance (SVR), mean arterial pressure (MAP), and decreased heart rate (HR) maximally at 1 minute but did not alter cardiac output (CO).[1] At higher doses (2.2–6.5 nmoles) NPY elicited a similar increase of SVR and MAP but produced a decrease of CO and MAP maximally at 5–20 minutes. To study the role of endogenous NPY in regulating cardiovascular function, NPY antiserum was administered ia to awake rats. A middle-directed polyclonal antiserum raised in rabbits injected with a glutaraldehyde-conjugated NPY human alpha-globulin complex administered to rats attenuated NPY-induced increase of MAP.[2] Unexpectedly, this NPY-NPY antiserum treatment resulted in a dramatic decrease of MAP and CO and an increase of HR and SVR. These changes of cardiovascular function were accompanied by an increase in plasma concentration of catecholamines and vasopressin. Treatment of animals with other hyperimmune sera did not produce this response. See FIGURES 1, 2, and 2.2.

The C-terminal analog of NPY, NPY(18–36), given ia did not produce any increase of MAP but did produce changes of MAP, CO, HR, and SVR in a temporal-qualitative pattern similar to that observed following treatment with NPY + NPY antiserum.[1, 3] Animals treated with NPY(18–36) exhibited a neurohumoral response, *i.e.*, increased plasma concentrations of catecholamines and vasopressin, similar to that observed following NPY + NPY antiserum.

Based on these data, the following conclusions are made:

1. NPY is capable of producing two types of cardiovascular responses. Type I response follows administration of low doses of NPY, occurs rapidly, and is associated with an increase of SVR and MAP. Type II response occurs maximally 5–15 min after administration of higher doses of NPY and is associated with a decrease of CO and MAP.
2. NPY antiserum attenuated the Type I response but augmented the Type II response, resulting in a dramatic decrease of CO and MAP followed by reflex tachycardia and neurohumorally mediated increase of SVR.

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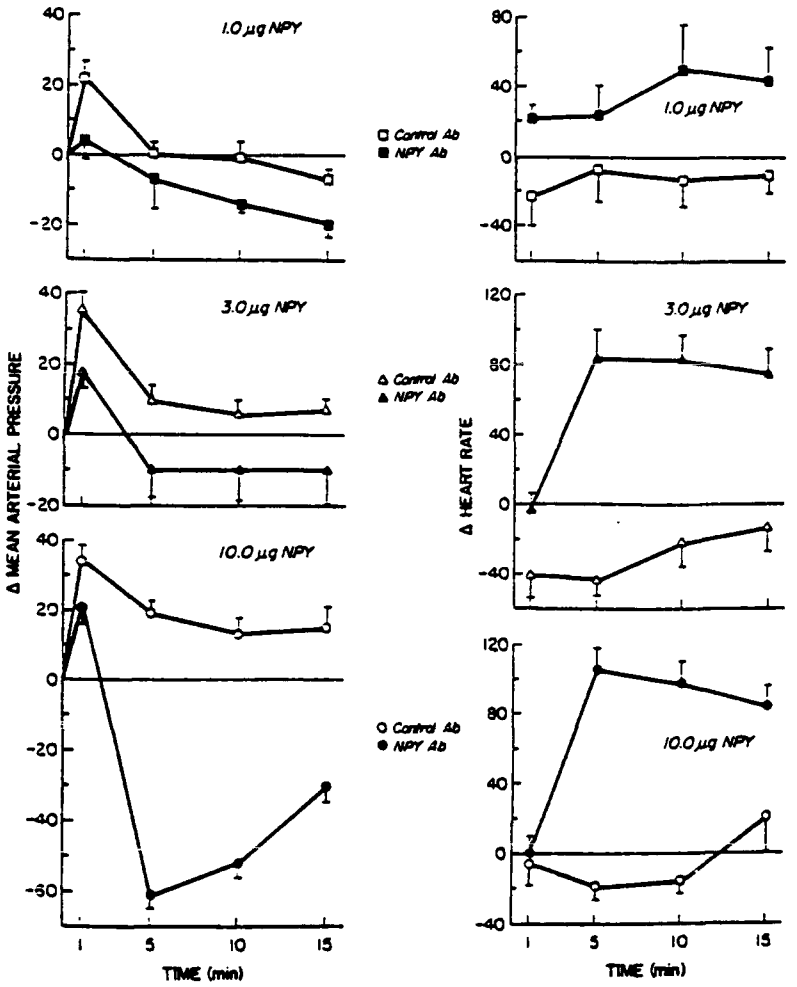


FIGURE 1. Effects of different doses of NPY on mean arterial pressure and heart rate in animals treated with NPY antiserum (500 μl) or hyperimmune serum (bombesin antiserum) given intra-arterially 10 min prior to peptide administration.

3. NPY(18–36) produced only a Type II response, resulting in decreases of CO and MAP with reflex tachycardia and neurohumorally mediated increase of SVR. Based on the postulated Y1 and Y2 receptor subtypes, it is possible that the Type I cardiovascular response is mediated by a Y1 receptor, and the Type II cardiovascular response is mediated by a Y2 receptor.

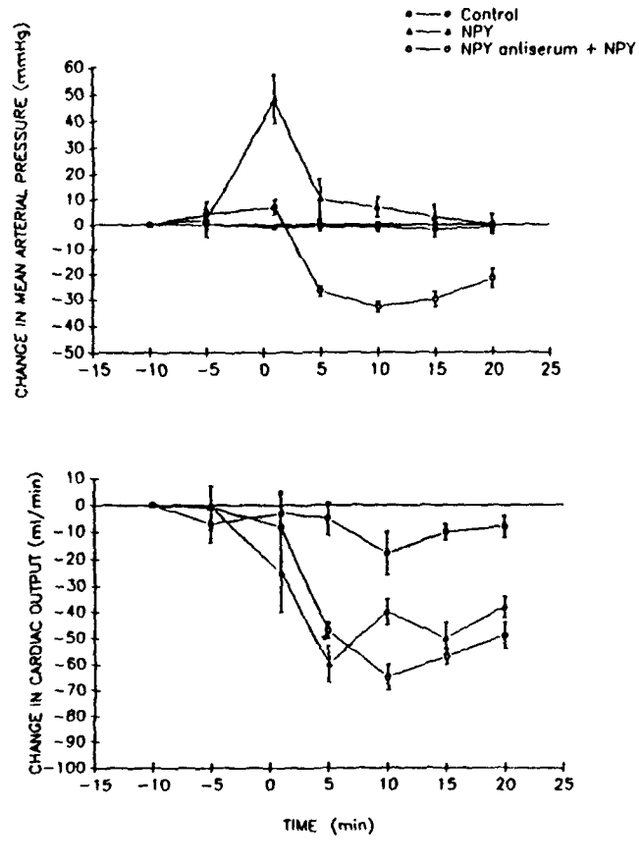
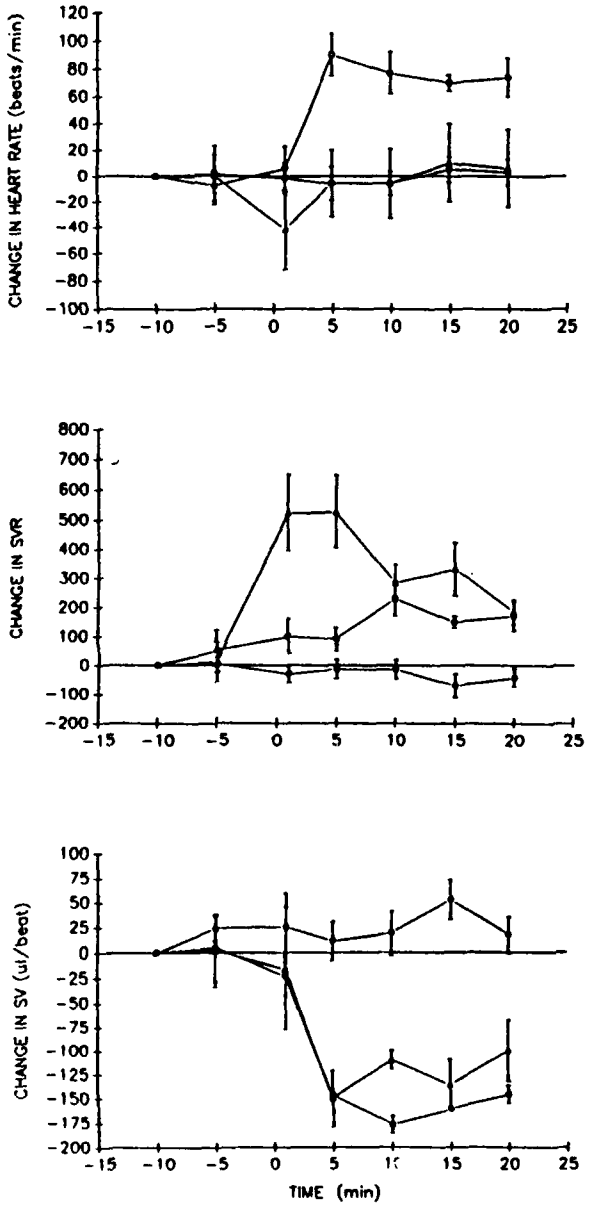


FIGURE 2. Effects of NPY and NPY antiserum + NPY on mean arterial pressure, cardiac output, heart rate, systemic vascular resistance, and strock volume. NPY *antiserum* (500 μl) was given at T = −10. NPY 2.2 nmoles was administered at T = 0.


CHANGE IN HEART RATE (beats/min)
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-15 -10 -5 0 5 10 15 20 25
CHANGE IN SVR
800
700
600
500
400
300
200
100
0
-100
-200
-15 -10 -5 0 5 10 15 20 25
CHANGE IN SV (ul/beat)
100
75
50
25
0
-25
-50
-75
-100
-125
-150
-175
-200
-15 -10 -5 0 5 10 15 20 25
TIME (min)

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Neuropeptide Y and Anglerfish Peptide Amide Inhibit Secretin-Stimulated Pancreatic Exocrine Secretion

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Neuropeptide Y (NPY) and anglerfish peptide amide (APY-NH_2) share substantial amino acid sequence homology with the pancreatic inhibitory peptides pancreatic polypeptide (PP) and peptide YY (PYY).[1] Immunoreactive NPY has been localized in pancreatic nerve fibers.[2] This rich NPY supply to the pancreas may indicate a role for NPY in the regulation of pancreatic secretion. This study was designed to evaluate the influence of exogenous NPY and APY-NH_2 on secretin-stimulated pancreatic exocrine secretion.

Four 25-kg dogs had Herrera pancreatic cannulae surgically implanted under general anesthesia. The dogs were allowed to recover for two weeks postoperatively and maintained their weight on standard dog chow. The animals were conditioned to stand in Pavlov harnesses. Studies were performed in randomized order at weekly intervals after an overnight fast.

At the beginning of each study the animals were given an intravenous infusion of secretin (0.5 μ/kg bolus followed by 0.5 μ/kg/hr) to attain maximal pancreatic stimulation. After plateau secretion was verified, one 30-minute basal period was measured. A separate infusion of NPY at 50, 100, or 100 pMol/kg/hr or APY-NH_2 at 100, 500, or 1000 pMol/kg/hr was performed for two 30-minute periods. One 30-minute recovery period concluded the study.

Pancreatic secretion was collected volumetrically in 30-minute aliquots. Pancreatic bicarbonate (HCO_3^-) was measured using an autoanalyzer (Radiometer, Copenhagen, Denmark). Protein was measured by the Lowry method.[3] Blood sampling was performed at 15-minute intervals for NPY and PYY radioimmunoassay.

RESULTS

Statistical analysis was performed using ANOVA with $p < 0.05$ accepted for significance. Results are expressed as mean ± SEM as percentage of basal secretion. NPY plasma levels averaged 420 ± 30 pg/ml basally. Significant elevations in circulating NPY were found in all three NPY doses averaging 670 ± 24 pg/ml, 1175 ± 93 pg/ml, and 3968

*Corresponding author.

TABLE 1. Effects of NPY on Pancreatic Exocrine Secretion

NPY Dose		Secretin Basal	Secretin + NPY 30′	Secretin + NPY 60′	Secretin 90′
50 pMol/kg/hr	Volume	100%	100 ± 21	117 ± 4	85 ± 12
	HCO_3^-	100%	87 ± 10	91 ± 8	91 ± 7
	Protein	100%	102 ± 4	110 ± 21	111 ± 12
100 pMol/kg/hr	Volume	100%	70 ± 13	63 ± 14	93 ± 14
	HCO_3^-	100%	67 ± 6*	65 ± 12*	71 ± 9
	Protein	100%	95 ± 8	83 ± 6	105 ± 7
1000 pMol/kg/hr	Volume	100%	69 ± 6*	53 ± 11*	94 ± 21
	HCO_3^-	100%	82 ± 6	64 ± 9*	84 ± 20
	Protein	100%	74 ± 18	83 ± 19	116 ± 27

*p <0.05 vs basal by ANOVA.

± 294 pg/ml, respectively. Basal PYY levels averaged 238 ± 20 pg/ml and were unaffected by NPY or APY-NH_2 infusion.

NPY significantly inhibited secretin-stimulated HCO_3^- secretion at the 100 and 1000 pMol doses. NPY inhibited volume secretion at only the 1000 pMol dose. No effect on protein secretion was demonstrated (TABLE 1). APY-NH_2 significantly inhibited pancreatic volume, HCO_3^-, and protein secretion at both 500 and 1000 pMol/kg/hr, an effect that extended into the recovery period (TABLE 2).

DISCUSSION

Our results demonstrate an inhibition of secretin-stimulated pancreatic exocrine secretion in a conscious animal model by both NPY and APY-NH_2. The evidence here supports a role for NPY in the physiologic control of pancreatic secretion. The marked effect of APY-NH_2 on pancreatic secretion suggests a possible clinical role for its use in acute pancreatitis.

TABLE 2. Effects of APY-NH_2 on Pancreatic Exocrine Secretion

APY-NH_2 Dose		Secretin Basal	Secretin + APY-NH_2 30′	Secretin + APY-NH_2 60′	Secretin 90′
100 pMol/kg/hr	Volume	100%	75 ± 14	64 ± 5	93 ± 9
	HCO_3^-	100%	69 ± 24	62 ± 23	78 ± 20
	Protein	100%	89 ± 13	97 ± 15	112 ± 17
500 pMol/kg/hr	Volume	100%	49 ± 7*	39 ± 3*	58 ± 6*
	HCO_3^-	100%	37 ± 3*	36 ± 4*	39 ± 8*
	Protein	100%	48 ± 6*	40 ± 4*	69 ± 7
1000 pMol/kg/hr	Volume	100%	42 ± 11*	49 ± 9*	74 ± 4
	HCO_3^-	100%	27 ± 7*	18 ± 4*	38 ± 8*
	Protein	100%	41 ± 12*	34 ± 7*	66 ± 2

*p <0.05 vs basal by ANOVA.

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Neuropeptide Y Does Not Mediate Ischemic Spinal Cord Injury following Aortic Cross-Clamping in Rabbits

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Paraplegia resulting from spinal cord ischemia during repair of thoracoabdominal aortic aneurysms is a significant clinical problem.[1] One postulated mechanism of neurologic damage includes posttraumatic ischemia secondary to persistent vasospasm. Neuropeptide Y (NPY), found in neural tissue, pia mater and cerebrospinal fluid (CSF), has been implicated in pathologic cerebral vasospasm.[2] This study examined the release of NPY into the CSF and blood in response to temporary occlusion of the suprarenal or infrarenal abdominal aorta in rabbits.

METHODS

New Zealand rabbits were randomized into three groups. In the first group the aorta was not clamped (n = 6); in the other two troups either the infrarenal aorta (n = 9) or the suprarenal aorta (n = 6) was clamped for 25 minutes. Animals were anesthetized with intravenous pentobarbital and the abdominal aorta exposed through a retroperitoneal approach. Blood and CSF (cisternal puncture) were collected before, during, and after cross-clamping the abdominal aorta. NPY was measured by a sensitive and specific radioimmunoassay developed in our laboratory.[3]

RESULTS

Plasma levels of NPY were not affected by temporary occlusion of either the suprarenal or infrarenal aorta (FIG. 1). NPY concentrations in the CSF were also unchanged except for a small decrease during aortic occlusion in the suprarenal group (FIG. 2). Lower extremity paralysis occurred in 70% of the infrarenal group, 83% of the suprarenal group, and 0% of the control group.

DISCUSSION

Little is known concerning the pathophysiology and treatment of spinal cord injury following nontraumatic ischemia. In models of cerebrospinal trauma, persistent vaso-

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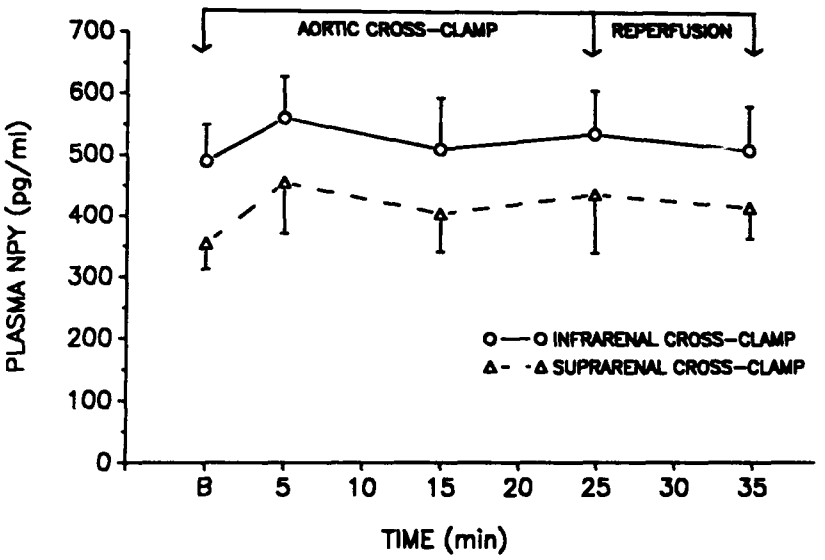

FIGURE 1. NPY plasma levels during aortic cross-clamping.

spasm is one of the mechanisms believed to contribute to neurologic damage. Recently, NPY has been implicated as a mediator of pathologic cerebral vasoconstriction. One of the models by which NPY may act is through release into the CSF. This has been

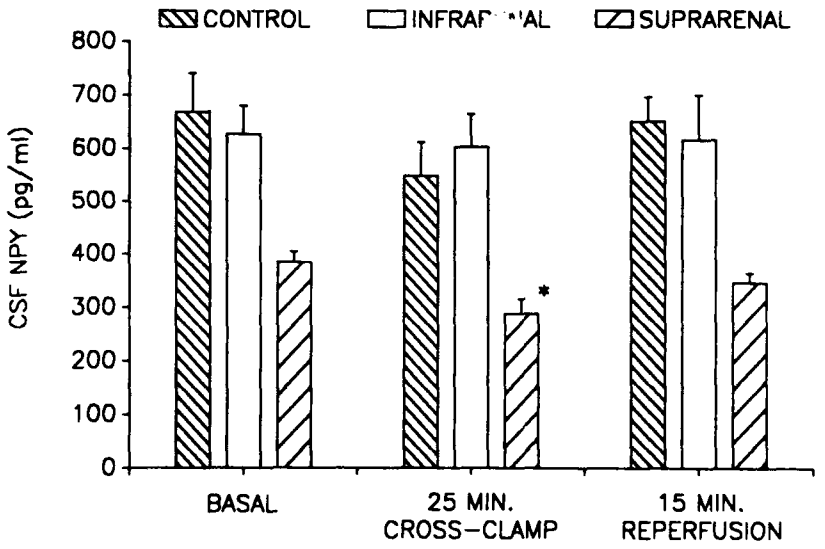

FIGURE 2. Cerebrospinal fluid NPY levels during aortic cross-clamping. *$p < 0.05$ versus basal by ANOVA.

previously described in association with centrally-stimulated hypertension, and after experimental subarachnoid hemorrhage.[4,5]

The present study demonstrates that NPY is not released into either the CSF or circulation as a result of cross-clamping the suprarenal or infrarenal abdominal aorta. Therefore, we are unable to suggest a role for CSF or plasma NPY in ischemic spinal cord injury following temporary aortic occlusion in rabbits. Our findings do not exclude that NPY may have a local effect on spinal cord blood flow independent of CSF release or circulating blood levels.

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Immunocytochemical Localization of Neuropeptide Y and Its C-Terminal Flanking Peptide in the Human Heart

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NPY is one of the most abundant and widespread peptides in the mammalian cardiovascular system.[1] Recently it was demonstrated that the predicted C-terminal flanking peptide of NPY (C-PON) is also widely distributed in both the central and peripheral nervous systems of several mammalian species including man, and has an identical distribution to NPY.[2] In the present study we investigated the localization and the possible coexistence of NPY and C-PON in the human heart using immunocytochemical techniques at both the light and electron microscopical levels. Immunofluorescence staining of cryostat sections and whole mount preparations revealed that the human heart is supplied by numerous nerve fibres containing NPY and C-PON immunoreactivity. NPY-immunoreactive nerve fibres were localized throughout the atrial and ventricular walls and exhibited a similar distribution pattern to nerves displaying C-PON immunoreactivity. NPY/C-PON-immunoreactive nerve fibres were more abundant in the atria than in the ventricles. In all four chambers NPY/C-PON-immunoreactive nerves were distributed throughout the myocardium, concentrated around small arteries and arterioles at the adventitial-medial border and formed an endocardial plexus (FIGS. 1a and 1b), which extended from the subendocardium to just beneath the endothelial lining. Intrinsic neuronal cell bodies in the human heart appeared to lack NPY/C-PON immunoreactivity. It was also observed that the number and distribution pattern of NPY/C-PON- and tyrosine hydroxylase-immunoreactive nerve fibres was very similar.

At the electron microscopical level the use of double immunogold staining techniques revealed that NPY and C-PON immunoreactivities were found consistently in large granular vesicles (70–100 nm in diameter), and both peptide immunoreactivities were colocalized to the same vesicles (FIG. 2) in axonal varicosities which usually also contained numerous small secretory vesicles (40–60 nm in diameter).

The findings demonstrate that NPY/C-PON-immunoreactive nerves are extensively distributed throughout the human heart and appear to largely represent postganglionic sympathetic neurones. At the ultrastructural level, the demonstration that NPY/C-PON

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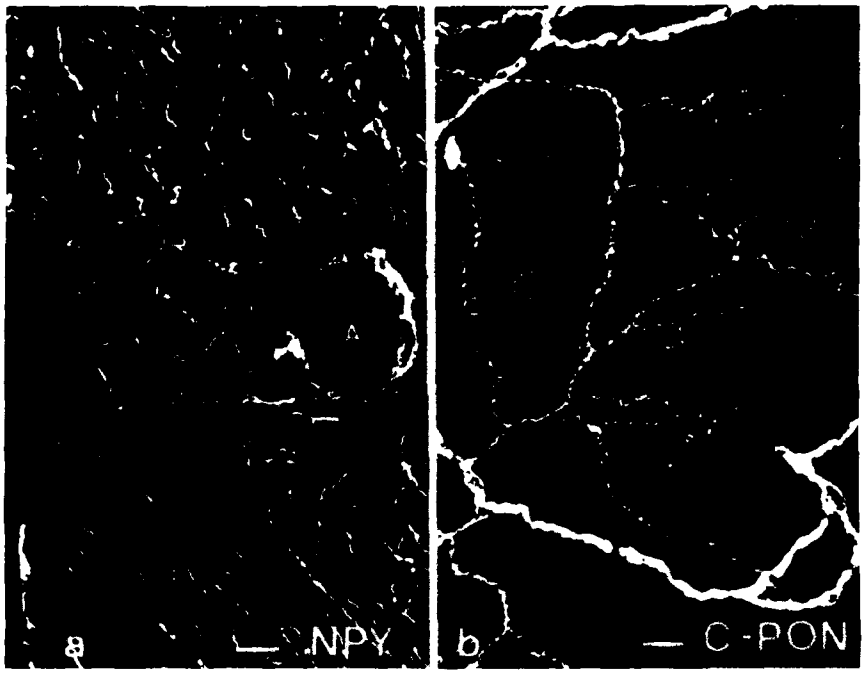

FIGURE 1. Immunofluorescence micrographs of a right atrial appendage cryostat section **(a)** and whole mount preparation of endocardium **(b)** immunostained for NPY (a) and C-PON (b). Dense network of NPY- and C-PON- immunoreactive nerve fibres and fascicles are present in the myocardium, around small arteries and arterioles (a) and in the endocardium (b). A: artery. Scale bar = 50 μm.

FIGURE 2. Axon varicosity in the human right atrial appendage myocardium. Colocalization of NPY and C-PON immunoreactivities to the same large granular secretory vesicles as demonstrated by a double immunogold staining procedure. NPY, 10 nm gold particles *(arrows)*. C-PON, 15 nm gold particles *(arrowheads)*. M: myocyte. Scale bar = 200 nm.

immunoreactivity was localized to large granular vesicles in presumed sympathetic nerve terminals supports the view that the storage of NPY and noradrenaline in two distinct subcellular compartments could facilitate the differential release of noradrenaline or NPY, the latter being preferentially released at high stimulation frequencies.[3]

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Neuropeptide Y and Nonadrenergic Mechanisms in the Sympathetic Vascular Control of the Nasal Mucosa

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INTRODUCTION

Coexistence of noradrenaline (NA) and neuropeptide Y (NPY)-like immunoreactivity (LI) was observed in the dense sympathetic innervation of resistance and capacitance vessels in the nasal mucosa of most species including man.[1]

A new *in vivo* model was developed in pentobabital anaesthetized pig which allowed parallel recordings of arterial blood flow (BF), volume of the nasal cavity (reflecting capacitance function V), nasal venous outflow (VOF) and superficial movement of blood cells (by mean of laser Doppler flowmetry (LDF) signal) of the nasal mucosa upon sympathetic nerve stimulation (SNS) and/or local intra-arterial injection of various pharmacological agents.[2]

AIMS OF THE STUDY

To evaluate the pig nasal mucosa content of NA and NPY-LI in control conditions, after reserpine pretreatment (± preganglionic denervation) and 2 weeks after sympathectomy.

To study the characteristics for NA and NPY release as revealed by overflow into the nasal mucosal venous effluent upon SNS *in vivo*.

To investigate the possible development of supersensitivity to vascular NA and NPY effects after sympathetic denervation of the pig nasal mucosa *in vivo*.

RESULTS

Reserpine pretreatment induced a marked depletion of both NA and NPY-LI in the pig nasal mucosa. The reduction of NA was not influenced by preganglionic denervation while the depletion of NPY then was prevented (TABLE 1). Two weeks after sympathectomy, the remaining NPY-LI seems to be of parasympathetic origin.[1]

SNS induced simultaneous reduction of BF, V, VOF and the LDF signal. After local i.a. pretreatment with the α-adrenoceptor antagonist phenoxybenzamine (1 mg kg^{-1}) the vascular responses to SNS were significantly attenuated but clear-cut reduction of BF, V,

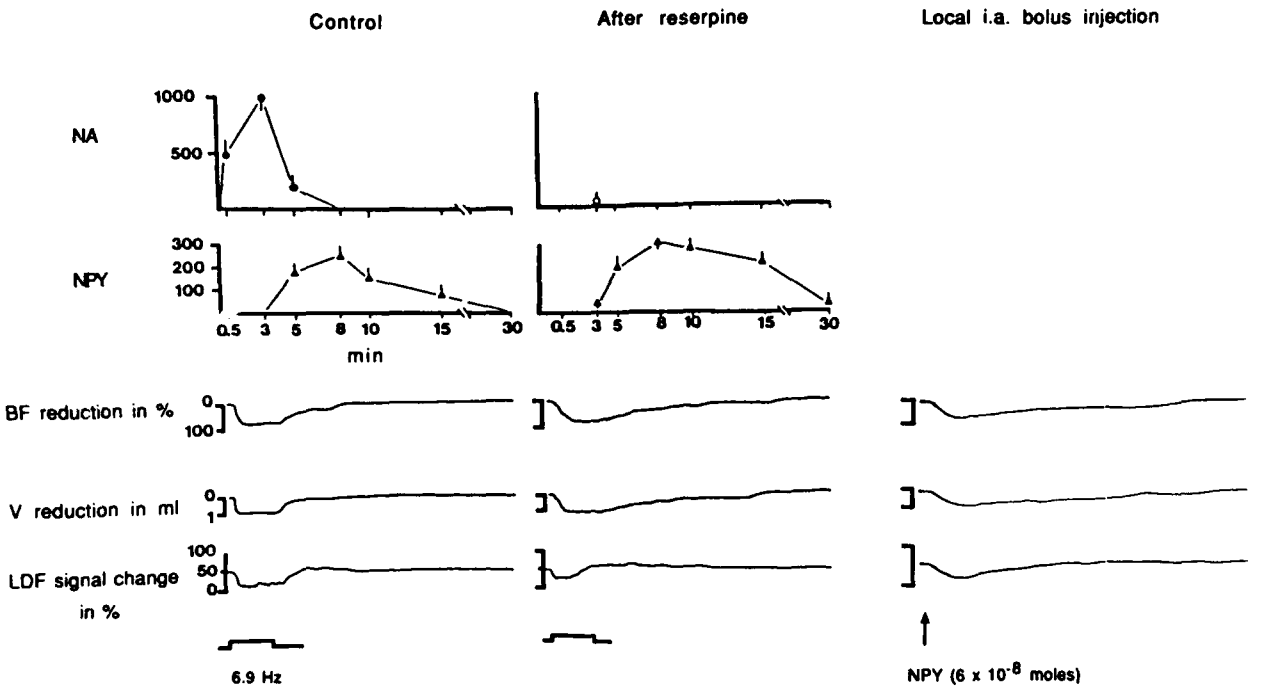

FIGURE 1. Time course for the SNS (6.9 Hz for 3 min) evoked NA (pmol min^{-1}) and NPY (fmol min^{-1}) overflow in the sphenopalatine vein (data are given as means values ± SEM, n = 5). Simultaneous recordings of nasal arterial blood flow reduction (BF, in %), nasal mucosal volume reduction (V, in ml) and laser Doppler flowmeter (LDF, in %) signal responses are shown for one experiment in a control animal and after reserpine treatment combined with preganglionic denervation. The vascular effects of local i.a. injection of NPY (6 x 10^{-8} moles) in control conditions are also shown.

VOF and the LDF signal remained even to single nerve impulses while the effects of exogenous NA were abolished.

A frequency-dependent increase of NA overflow was observed in the nasal venous effluent upon SNS in control pigs, whereas detectable release of NPY-LI occurred only at high frequency.

Slowly developing, frequency-dependent and long-lasting vascular responses remained (up to 80% of control) upon SNS in reserpinized and decentralized animals. In these animals, the NA overflow was abolished while release of NPY-LI was enhanced. Exogenous NPY mimicked the reserpine-resistant SNS-evoked vascular responses (FIG. 1).

Two weeks after sympathectomy, an increased sensitivity to the vasoconstrictor effects of NA and NPY was observed (not shown). The development of *in vivo* supersensitivity to NPY further suggests a physiological role of this peptide as a nonadrenergic mediator in the sympathetic vascular control of the nasal mucosa. Although NA is likely to mediate most of the sympathetic vascular responses to low frequency SNS, some nonadrenergic effects remain even to single impulse stimulation. The characteristic "NPY-like" vascular responses which remained following SNS in spite of adrenoceptor blockade or reserpine-induced NA depletion and the parallel NPY-LI overflow, suggest that NPY could mediate the nonadrenergic component of sympathetic nasal vasoconstriction.

TABLE 1. Pig Nasal Mucosal Content of NA and NPY-LI[a]

	NA (nmol/g)			NPY-LI (pmol/g)		
	Non-stimulated	Reduction in %	SNS	Non-stimulated	Reduction in %	SNS
Control	14.00 ± 1.60		9.7 ± 1.0	11.4 ± 1.2		9.1 ± 1.9
After reserpine	0.09 ± 0.01	99**	—	6.3 ± 0.5	44*	—
After reserpine and preganglionic denervaton	0.08 ± 0.02	99**	0.06 ± 0.02	14.5 ± 1.5		7.8 ± 2.0*
2 weeks after sympathectomy	0.10 ± 0.03	99**	—	3.1 ± 0.10	74**	—

[a]Data (means ± SEM, n = 8) from control animals are compared with biopsies obtained from animals pretreated 24 h before with reserpine (1 mg kg^{-1}, i.v.), with or without preganglionic denervation and finally 2 weeks after surgical sympathectomy. For comparison, the tissue content after a course of sympathetic nerve stimulations (SNS) are also shown. *p <0.05; **p <0.01; ***p <0.001.

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Neuropeptide Y Causes Nonadrenergic Vasoconstriction in the Eye, Brain, and Some Other Tissues in Rabbits

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The eye has a rich innervation of sympathetic nerve fibers from the superior cervical ganglion and like sympathetic nerve fibers to many other tissues, they are immunoreactive to neuropeptide Y (NPY). Although the density of the NPY-fibers differs between species, the distribution pattern seems to be about the same in most species. The iris dilatator muscle and the blood vessels of the uvea (choroid, iris and ciliary body) have a rich innervation, but there are NPY-fibers present also in the ciliary processes and outflow apparatus.[1] The innervation pattern suggests that NPY may have effects on the pupil size, ocular blood flow and aqueous humor dynamics. In the present study, the effects of NPY on regional blood flows in the eye and other tissues were studied in the rabbit.

The experiments were made on albino rabbits under urethane anesthesia (7 ml/kg b.w. of a 25% solution). All animals were tracheotomized and artificially ventilated. In eight rabbits, uveal blood flow (UBF) was determined by direct measurement of the flow from a cannulated vortex vein simultaneously with registration of the mean arterial blood pressure (MABP) and the intraocular pressure (IOP). The uveal vascular resistance (UVR) was calculated as (MABP-IOP)/UBF.[2] Regional blood flows were measured with radioactively labelled microspheres[3] (15 μm) in twenty-five rabbits. Two different experimental series were made; one with and one without α-adrenoceptor blockade (phenoxybenzamine 50 mg/kg b.w.). Blood flow determinations were made before and at 2 and 10 min after the start of i.v. infusion of NPY, 120 pmol/kg x min. The cervical sympathetic nerve was sectioned on one side in all experiments. Blood flows during the NPY-infusion were compared with blood flows under control conditions by Student *t* test. Mean values and SE are given.

Intravenous infusion of increasing doses of NPY (7.5–120 pmol/kg x min) caused a dose-dependent increase in the UVR. Near maximal effect (70% increase) was achieved with 120 pmol/kg x min. In the microsphere experiments without α-adrenoceptor blockade, the NPY-infusion had no significant effect on the MABP, but cardiac output was significantly decreased at 10 min. After 2 min of NPY-infusion, local blood flows were markedly decreased in the kidneys, spleen and adrenal glands (FIG. 1A). There were also significant blood flow reductions (20–60%) in different parts of the gastro-intestinal tract. In these tissues there was no further decrease in blood flow at 10 min (FIG. 1A) and the effects of NPY were not affected by α-adrenoceptor blockade (FIG. 1B). In the eye the effect of NPY was moderate at 2 min, but at 10 min there were marked blood flow reductions in all parts of the uvea. The blood flow reductions on intact and sectioned side respectively were for the choroid 55 ± 5 and 56 ± 4% ($p \leq 0.001$), ciliary body 53 ± 5 and 52 ± 6% ($p \leq 0.001$) and iris 33 ± 7 and 28 ± 8% ($p \leq 0.01$). The salivary glands, tongue and masseter muscle responded in a similar way as the eye; moderate effect at 2

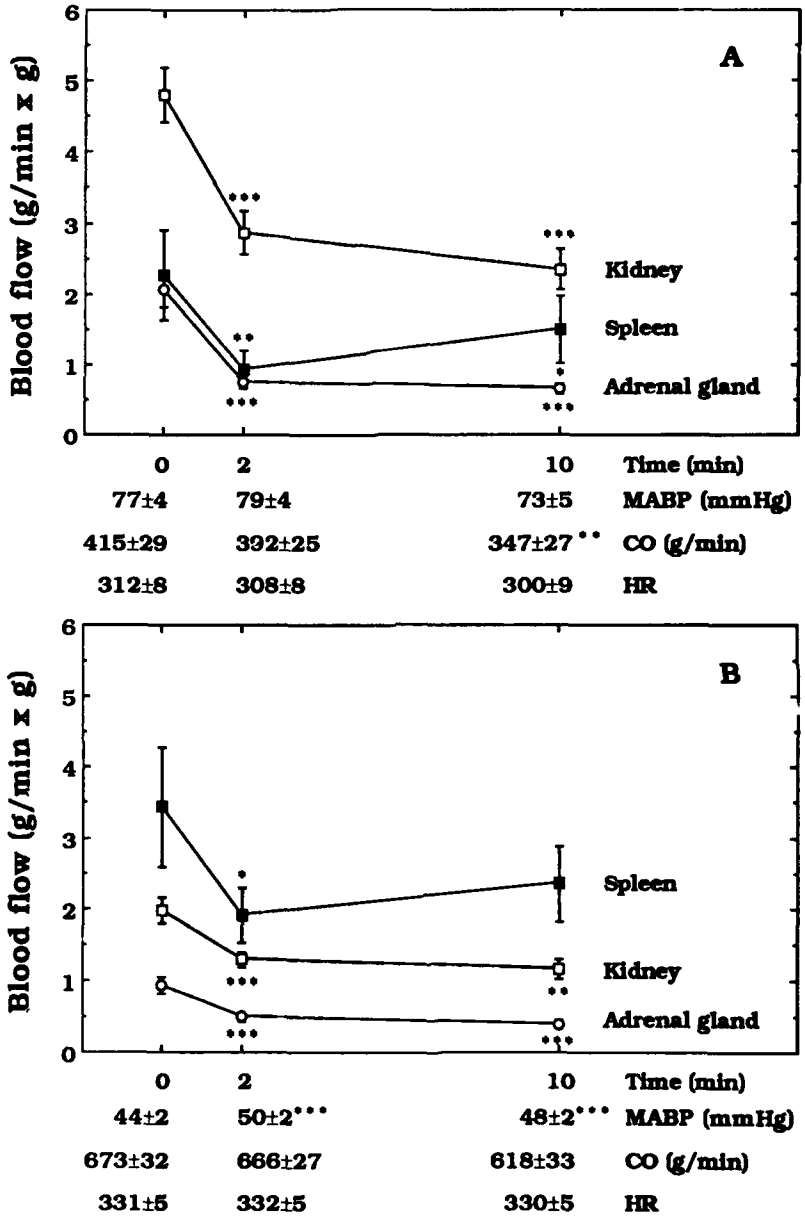

FIGURE 1. Mean arterial blood pressure (MABP), cardiac output (CO), heart rate (HR) and local blood flows in the kidney, spleen and adrenal gland before and at 2 and 10 min after the start of i.v infusion of NPY, 120 pmol/kg x min. **(A)** Experiments without α-adrenoceptor blockade (n = 12). **(B)** Experiments with α-adrenoceptor blockade (n = 13). $*p \leq 0.05$; $**p \leq 0.01$; and $***p \leq 0.001$.

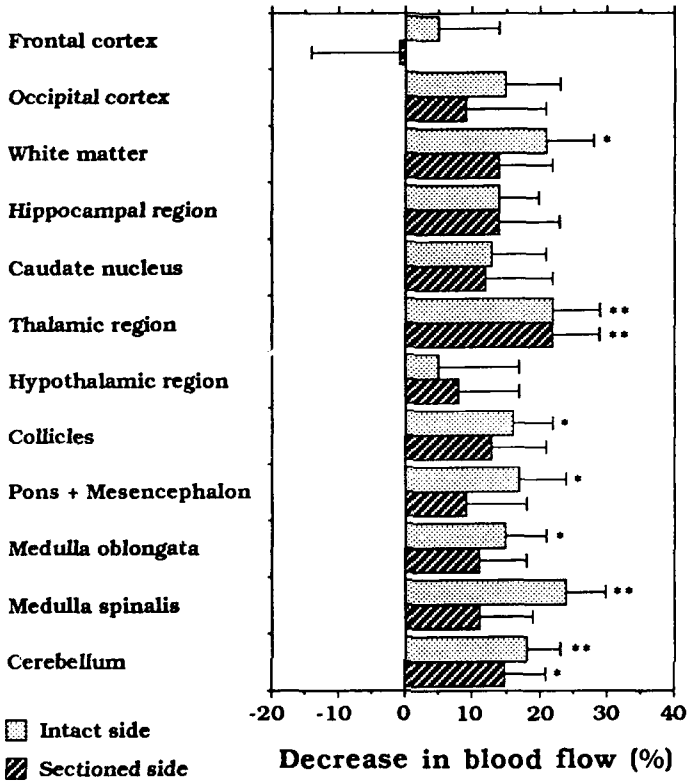

FIGURE 2. Decreases in regional cerebral blood flows at 10 min after the start of i.v. infusion of NPY, 120 pmol/kg x min, in experiments without α-adrenoceptor blockade (n = 9, except for cerebellum for which n = 12). *$p < 0.05$ and **$p < 0.01$.

min, but marked at 10 min (30–70% decrease). In these tissues, the initial blood flow and the response to NPY were larger on the sectioned side. The blood flow reductions in the eye and other facial tissues seemed to be less in the experiments with α-adrenoceptor blockade, which partly could be due to the fact that NPY increased the MABP in these experiments (FIG. 1B). Calculation of the change in vascular resistance showed that there was no large difference in the response to NPY between the two experimental series. At 10 min regional cerebral blood flows were decreased by 15–20% (FIG. 2). In the choroid plexus, pineal and pituitary gland, local blood flows were decreased by 30–60%.

The results of the present study show that NPY has marked effects on regional blood flows in the rabbit and gives further support to the suggestion that NPY may significantly contribute to the sympathetic vasoconstriction in the rabbit uvea.[1]

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Effects of Neuropeptide Y and Noradrenaline on the Uterine Artery

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INTRODUCTION

Many peripheral sympathetic nerves, including those of the female reproductive tract, contain both noradrenaline (NA) and neuropeptide Y (NPY).[1] Both substances are vasoactive, and are released upon nerve stimulation.[2] During pregnancy, there is a decrease of uterine sympathetic nerves.[3] We have studied the effects of NPY and NA *in vitro* on isolated human nonpregnant uterine arteries, and also in a situation where the uterine sympathetic nerves have degenerated, in the uterine circulation of the pregnant guinea pig *in vivo*.[4]

METHODS

Segments of human uterine arteries and veins were obtained during elective surgery, and mounted in oxygenated Krebs-Ringer buffer in organ baths connected to a Grass FT03C transducer and model 7 polygraph. *In vivo*, local arterial blood pressure was measured in term pregnant guinea pigs through a cannulated side branch of the uterine artery. Blood flow was measured by ultrasound Doppler velocimetry.[5]

RESULTS

In the isolated human uterine artery, NPY had no effects per se. However, NPY in higher concentrations (10^{-7} M) often, but not always, enhanced the contractile effect of NA (FIG. 1). In the human uterine vein, NPY increased basal tone and induced spontaneous contractions. The contractile effect of nerve stimulation was also enhanced by NPY. In the pregnant guinea pig uterus, NPY increased blood pressure, but had no significant effect on blood flow, *i.e.*, the net effect was an increased vascular resistance. NPY (10^{-7} M) was more potent than NA (10^{-7} M) in increasing blood pressure (FIG. 2). The effects of NA were reduced or abolished by phentolamine, whereas the effects of NPY were not affected by phentolamine.

[a]Supported by the Swedish Medical Research Council (14X-7164).

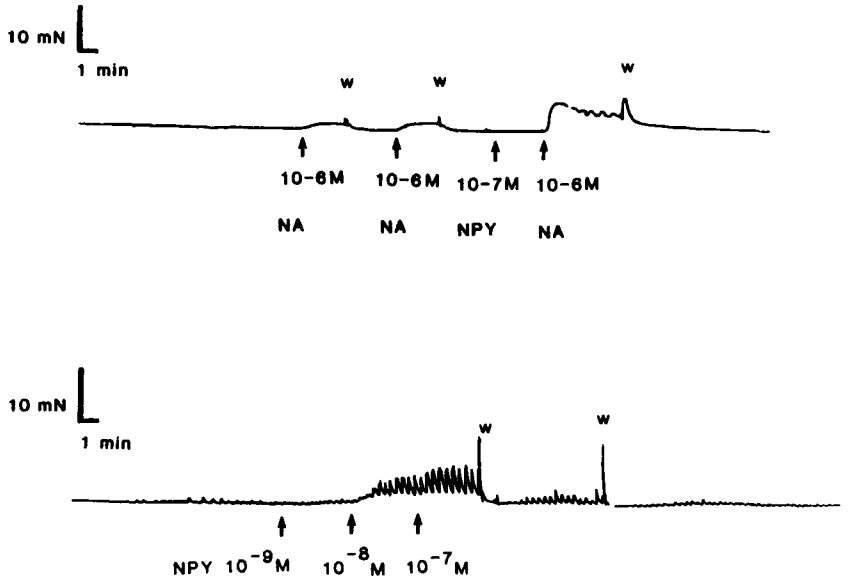

FIGURE 1. *Upper trace:* Effects of NPY and NA on human uterine artery *in vitro*. NPY (10^{-7} M) has no intrinsic effect, but appears to enhance the contraction caused by NA (10^{-6} M). w = wash. *Lower trace:* Effect of NPY on human uterine vein *in vitro*. There is an increased tone at 10^{-9} M, spontaneous contractions appear at 10^{-8} M, and an increase in amplitude appears at 10^{-7} M, and disappears after wash (w).

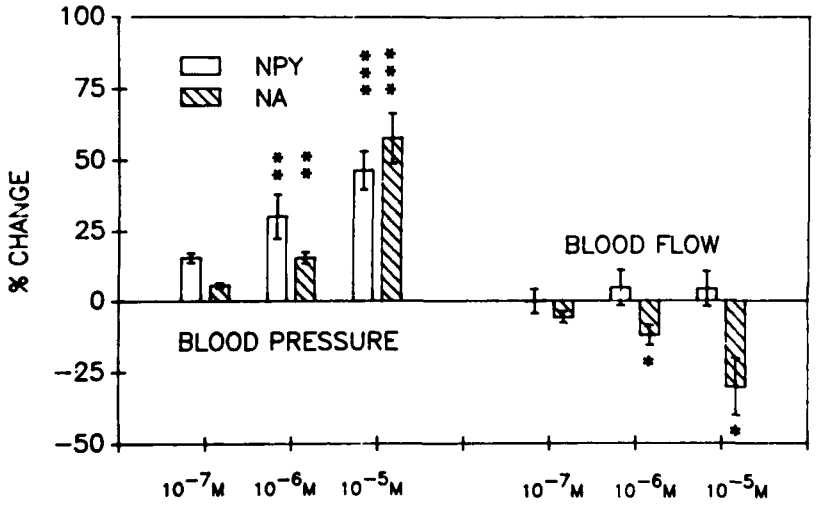

FIGURE 2. Effects of NPY and NA on blood pressure (*left panel*) and blood flow velocity (*right panel*) in the pregnant guinea pig artery. On the abscissa, concentrations of NPY and NA are given (10^{-7}–10^{-5} M). On the ordinate, percentage changes in blood pressure and blood flow velocity as compared to control values (averages obtained 2 min before the infusion). *Open bars* = NPY, *solid bars* = NA. *** = $p < 0.001$; ** = $p < 0.05$; * = $p < 0.05$.

DISCUSSION

The results show that NPY has no vasoconstrictor effects per se in the nonpregnant human uterine artery, and moderate vasoconstrictor effects in the human uterine vein. In the pregnant guinea pig, local infusion of NPY gave an increased blood pressure but no effect on blood flow velocity. Taken together, the results indicate that NPY cooperates with NA to cause an increased vascular resistance. The effect of NPY appears to be α_1-adrenoceptor resistant. Both NPY and NA may thus be involved in the control of the uterine circulation, both before and during pregnancy.

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Reduced Inhibitory Actions of Sympathetic Stimulation and Exogenous Neuropeptide Y on Cardiac Vagal Action in the Presence of Peptide YY

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It has been proposed that neuropeptide Y (NPY) is released by stimulation of the cardiac sympathetic nerves in the anaesthetized dog and is responsible for a subsequent prolonged inhibition of cardiac vagal action by inhibiting acetylcholine release.[1] Testing this proposal has been hindered by lack of a specific antagonist to NPY.

Another approach, however, is to use a peptide which has similar but more potent NPY-like action as a 'superagonist.' Peptide YY (PYY), a related peptide also inhibits cardiac vagal action but is more powerful than NPY and has a longer duration of action.[2] It is postulated that when PYY is occupying receptors on vagal nerve terminals, nerve-released NPY will be either unable to produce an effect, because it cannot gain access to the receptors, or it will displace PYY from at least some receptors and, being less powerful than PYY, will lessen the preexisting vagal attenuation.

The following ratio was measured in anaesthetised dogs before and after PYY administration:

$$R = \frac{\Delta PI \text{ after sympathetic stimulation or NPY}}{\Delta PI \text{ before sympathetic stimulation or NPY}}$$

ΔPI is the increment in pulse-interval (the period between successive beats of the heart) evoked in response to a standard vagal simulus. R becomes <1 when sympathetic nerve stimulation or exogenous NPY inhibit the cardiac slowing effect of the vagus. R was measured before and after PYY administration.

R was measured for sympathetic stimulation in nine dogs. In all of these there was less inhibition of vagal action on pulse interval (*i.e.*, R was greater) in the presence of PYY than in its absence. Indeed, in 5 of the dogs, R, which was always <1 in control circumstances, became >1 in the presence of PYY. This indicates that in the presence of PYY sympathetic stimulation was associated with enhancement rather than inhibition of vagal action on pulse interval (FIG. 1). Such an effect could occur if the less potent transmitter NPY were released from sympathetic nerves, displacing PYY from receptors on vagal nerve terminals, and so relieving the vagal inhibition established in the presence of PYY.

R was measured for NPY administration in 3 dogs, in the absence then presence of PYY. In all of these R was increased in the presence of PYY, and in 2 of them R was equal to 1, indicating a failure of NPY to inhibit cardiac vagal action in the presence of

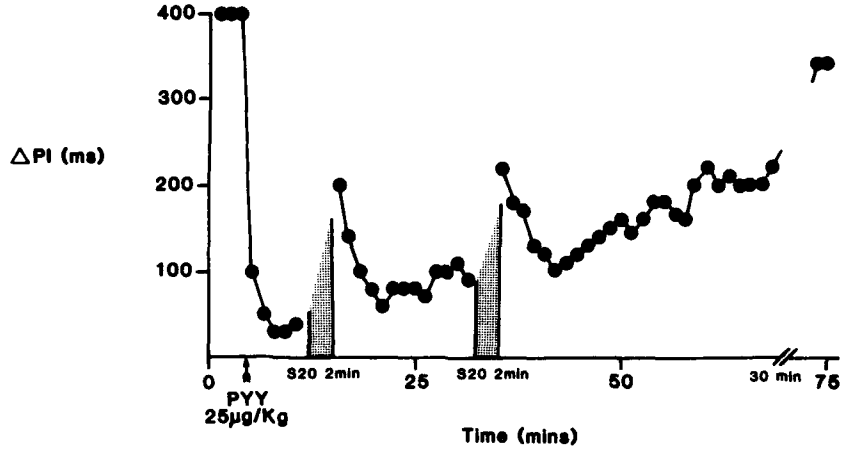

FIGURE 1. Electrical stimulation of the cardiac sympathetic nerve (S20) inhibits cardiac vagal action. In the presence of PYY stimulation of the cardiac sympathetic nerve does not further inhibit cardiac vagal action; in this example the inhibitory action of cardiac sympathetic nerve stimulation is transiently relieved.

PYY. Again, displacement of PYY from receptors by the less potent NPY would account for the findings.

These results therefore support the proposal that it is NPY released during sympathetic stimulation that is responsible for the prolonged attenuation of vagal action.

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Pre- and Postjunctional Actions of Neuropeptide Y and Related Peptides

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The effects of neuropeptide Y (NPY) and related peptide fragments on arterial blood pressure (BP) and cardiac vagal action were studied in vagotomised anaesthetised rats. An increase in BP was used as a measure of postjunctional action of peptide (direct vasoconstrictor and/or constrictor-potentiating), and a change in cardiac vagal action was taken as a measure of prejunctional activity. Three analogs of NPY were studied in detail in two series of experiments.

In the first series, the effect of equimolar doses of NPY 13-36 and a 'stabilised' NPY 13-36 (ANA-NPY[1]) were compared with whole NPY. The results are listed in TABLE 1. It can be seen that both 13-36 fragments have significant pre- and postjunctional activity. ANA-NPY, the stabilised fragment, has similar prejunctional activity to whole NPY, but is less potent postjunctionally. The findings are consistent with there being more than one NPY receptor type, but do not support the basis of distinction of the prejunctional one being its failure to be activated by the 13-16 fragment.[2]

In the second series of experiments the activity of the C-terminal fragment NPY 18-36 was compared with whole NPY in 18 rats. NPY 18-36 has both pre- and postjunctional activity but is significantly less active than NPY 1-36 at both sites. In *intact* anaesthetised rats in contrast with the findings in vagotomised rats, however, NPY 18-36 was found to have a prominent depressor action. This was especially notable at higher doses of peptide, and was typically preceded by a small, transient pressor response. This depressor action was greatly attenuated, or absent, after vagotomy (FIG. 1). The results help explain the findings of depressor effects of NPY 18-36 reported by Boublik and associates[3] in rats with intact vagi. The dependence of the effects on the vagus suggest that NPY 18-36 acts by activating sensory nerves that travel in the vagus and produce their depressor actions reflexly.

TABLE 1. Pre- and Postjunctional Actions of NPY and Related Peptides

	NPY 1-36	NPY 13-36	ANA-NPY
BP (mm Hg): postjunctional	71 ± 4	40 ± 1	30 ± 3
% Vagal inhibition: prejunctional	91 ± 3	52 ± 9	86 ± 7

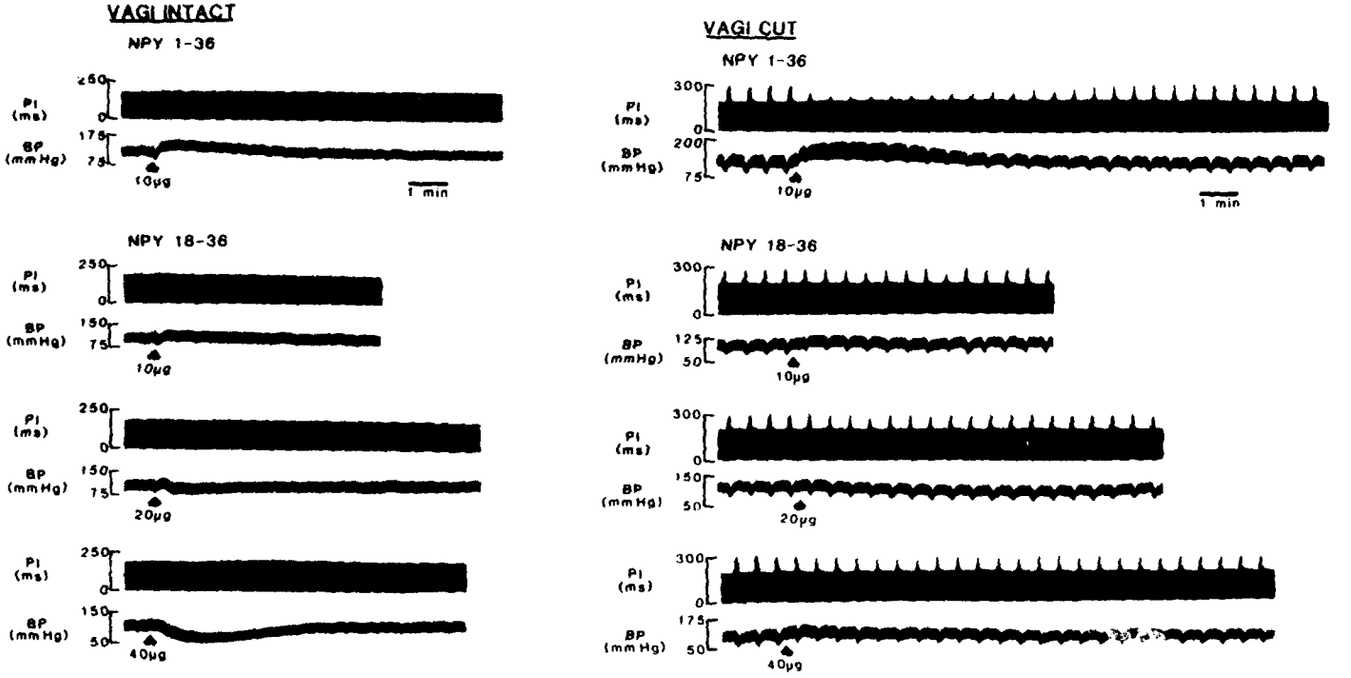

FIGURE 1. Records from an anaesthetised rat show in each panel pulse interval (PI, *top trace*) and arterial blood pressure (BP, *bottom trace*) before (*left*) and after vagotomy (*right*). After vagotomy the peripheral end of the right vagus nerve was given a standard electrical stimulus every 30 seconds to increase pulse interval. The top panels show the effect of NPY 1-36 (10 μg i.v.) on pulse interval and blood pressure. The bottom three panels on both sides show the effect of NPY 18-36 (10 μg, 20 μg, 40 μg, i.v.) on blood pressure and pulse interval. Note neither NPY 1-36 nor NPY 18–36 has any direct effect on pulse interval (*left panels*). Note also that increasing doses of NPY 18-36 cause a fall in blood pressure with vagus nerves intact (*left*), but modest rises in blood pressure after vagotomy (*right*).

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Neuropeptide Y—A Marker for Noradrenaline Exocytosis

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In ischemia adrenergic excess stimulation of the myocardium is accompanied by deleterious consequences such as the occurrence of fatal arrhythmias and the accleleration of cell damage.[1] Local release of noradrenaline in the energy-depleted heart can be mediated by two distinct release mechanisms, either by exocytosis or by a nonexocytotic release mechanism. We now provide evidence that neuropeptide Y (NPY), which is colocalized with noradrenaline in cardiac postganglionic sympathetic nerve fibres, can be utilized as a marker for exocytotic noradrenaline release and therefore differentiates between both release mechanisms.[2]

METHODS

All experiments were performed in guinea pig perfused hearts, which were prepared according to a modified Langendorff technique and perfused with Krebs-Henseleit buffer at a constant flow rate of 7 ml/min/g heart weight.[3] Two models of energy depletion were employed: 1. anoxia (pO_2 less than 5 mmHg);[2,4,5] 2. cyanide intoxication plus glucose-free perfusion.[4,5] In the coronary venous effluent noradrenaline and NPY were determined by HPLC[4,5] and RIA,[3] respectively.

RESULTS AND DISCUSSION

Central sympathetic stimulation is accompanied by a concomitant overflow of noradrenaline and NPY from the heart.[6] The corelease of both transmitters induced by electrical stimulation of postganglionic sympathetic nerve fibres, veratridine or nicotine requires the presence of extracellular calcium and has therefore been attributed to exocytosis.[3,6] In contrast, noradrenaline efflux evoked by the indirectly acting sympathomimetic tyramine is independent from the extracellular calcium concentration, the activation of N-type calcium channels and the stimulation of protein kinase C, and is not accompanied by any significant overflow of NPY (FIG. 1). The tyramine-induced efflux of noradrenaline can be described by a nonexocytotic release mechanism, consisting of a two step process: In a first step noradrenaline is lost from storage vesicles, resulting in increased axoplasmic noradrenaline concentrations. In a second, rate-limiting step noradrenaline is transported across the axolemm into the synaptic cleft using the neuronal catecholamine re-uptake ($uptake_1$) in reverse of its normal transport direction. The latter

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mechanism also accounts for the local metabolic release of noradrenaline occurring in the energy-depleted heart.[1] The local metabolic release of noradrenaline during anoxia,[2] cyanide intoxication (unpublished observations), and ischemia[7] is not accompanied by overflow of NPY and is quite distinct from exocytosis, as summarized in TABLE 1: Uptake$_1$-blockade (*e.g.*, by desipramine) increases exocytotic and suppresses nonexocytotic noradrenaline overflow from the heart. Whereas exocytotic overflow of noradrenaline and NPY is modulated by presynaptic receptors,[3-6] *nonexocytotic* noradrenaline release occurs independent from presynaptic regulation.[1]

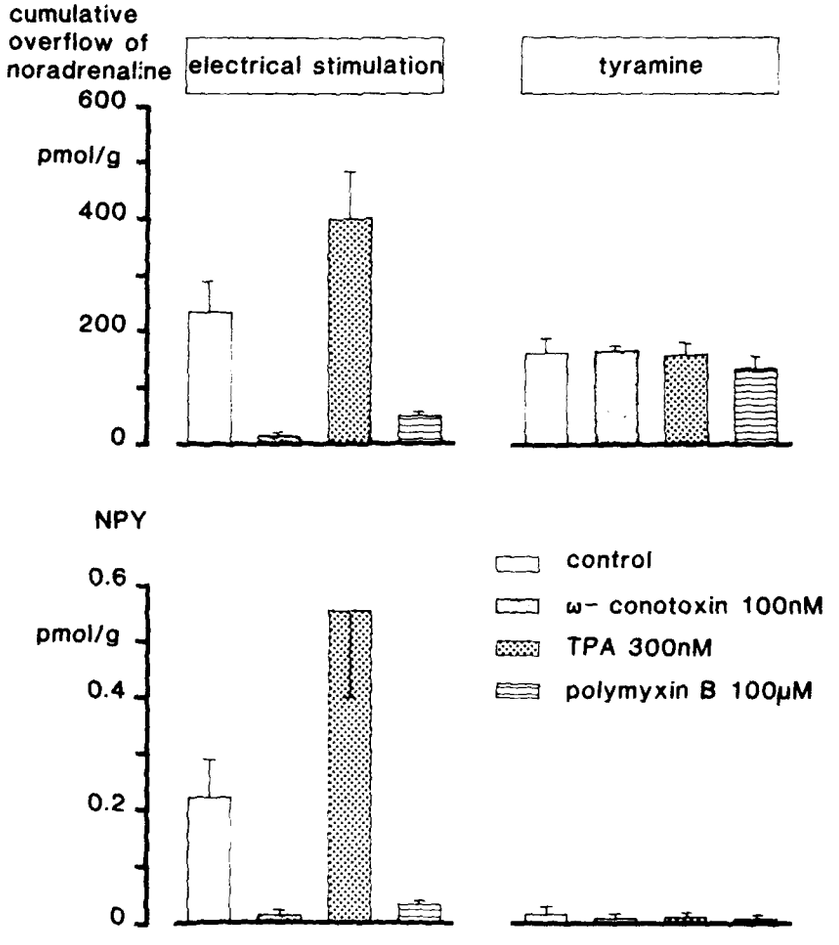

FIGURE 1. Effect of blockade of N-type calcium channels (by ω-conotoxin), and of activation (by phorbol-12-myristate-13-acetate (TPA)) or inhibition (by polymyxin B) of protein kinase C on the overflow of noradrenaline and neuropeptide Y (NPY) from the guinea pig perfused heart evoked by electrical stimulation (12 Hz; 1 min) of cardiac sympathetic nerve fibres (*left panel*) or by 10-min infusion of tyramine (*right panel*). All hearts were perfused with a calcium-containing Krebs-Henseleit buffer; ^{2} n = 4–6 in each group; mean ± SEM.

TABLE 1. Characteristics of Nonexocytotic Noradrenaline Release in the Guinea Pig Perfused Heart during Energy Depletion or Tyramine Administration: Comparison with Exocytotic Noradrenaline Release

	Exocytotic Release	Nonexocytotic Release
Central sympathetic stimulation	+	−
Concomitant overflow of NPY	+	−
Modulation by presynaptic receptors	+	−
Dependence from extracellular calcium	+	−
Inhibition by uptake$_1$-blockade	−	+

During early stages of ischemia, both release mechanisms play a role in the adrenergic overstimulation of the heart.[1] Hence, simultaneous measurement of noradrenaline and NPY allows to assess the relative contribution of exocytotic and nonexocytotic transmitter release and may help to determine therapeutic strategies to suppress the local catecholamine excess.

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Common Features of Neuropeptide Y and Noradrenaline Release in Guinea Pig Heart

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Neuropeptide Y (NPY) is colocalized with catecholamines in postganglionic sympathetic nerve fibres and the adrenal medulla. Furthermore, sympathetic stimulation has been demonstrated to be accompanied by a corelease of NPY and noradrenaline in animal models[1] and in man.[2] This short review will focus on our most recent studies characterizing the common features of the exocytotic release of NPY and noradrenaline from guinea pig *in situ* perfused hearts with intact sympathetic innervation.

METHODS

For *in situ* perfusion (constant flow of 7 ml/min/g heart weight: Krebs-Henseleit buffer) the guinea pig hearts were prepared according to a modified Langendorff technique. In addition, the left stellate ganglion was carefully dissected free for electrical stimulation (12 Hz, 1 min) of cardiac sympathetic nerve fibres.[3] In the coronary venous effluent NPY and noradrenaline were determined by RIA[3] and HPLC,[4] respectively.

RESULTS AND DISCUSSION

The common features of stimulation-evoked release of NPY and noradrenaline are summarized in TABLE 1. The corelease of both transmitters evoked by electrical stimulation of the left stellate ganglion is positively related to the stimulation frequency[3] and to the extracellular calcium concentration,[5] and is regulated by presynaptic receptors, such as alpha$_2$-adrenoceptors, adenosine A_1-receptors, and angiotensin II-receptors, in a parallel manner. By means of a negative feedback mechanism both noradrenaline and possibly NPY reduce their corelease by interference with presynaptic alpha$_2$- and NPY-receptors, respectively. As observed in guinea pig heart[3] and pig spleen,[1] blockade of neuronal amine re-uptake (uptake$_1$) by desipramine or nisoxetine differentially affects the transmitter overflow. Uptake$_1$-blockade increases noradrenaline and reduces NPY overflow. This phenomenon can be explained by an increased stimulation of inhibitory presynaptic alpha$_2$-adrenoceptors induced by the elevated local concentrations of noradrenaline.[3]

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TABLE 1. Common Features of the Stimulation-Evoked Exocytotic Corelease of Neuropeptide Y (NPY) and Noradrenaline (NA) in the Guinea Pig Perfused Heart[a]

		NPY	NA
General features			
Frequency-dependence		+	+
Calcium-dependence		+	+
Presynaptic receptors			
$Alpha_1$-adrenoceptor	prazosin (I)	–	–
$Alpha_2$-adrenoceptor	yohimbin (I)	↑	↑
	B-HT 920 (S)	↓	↓
NPY-receptor	NPY (S)	?	↓
Adrenosine A_1-receptor	cyclohexyladenosine (S)	↓	↓
Angiotensin II-receptor	angiotensin II (S)	↑	↑
Amine uptake			
$Uptake_1$	desipramine (I)	↓	↑
	nisoxetine (I)	↓	↑
Calcium channels			
N-type	ω-conotoxin (I)	↓	↓
	cadmium chloride (I)	↓	↓
L-type	felodipine (I)	–	–
Second messengers			
Protein kinase C	TPA (S)	↑	↑
	polymyxin B (I)	↓	↓

[a](I) = inhibitory; (S) = stimulatory; + = change; – = no change; ↓ = decrease; ↑ = increase.

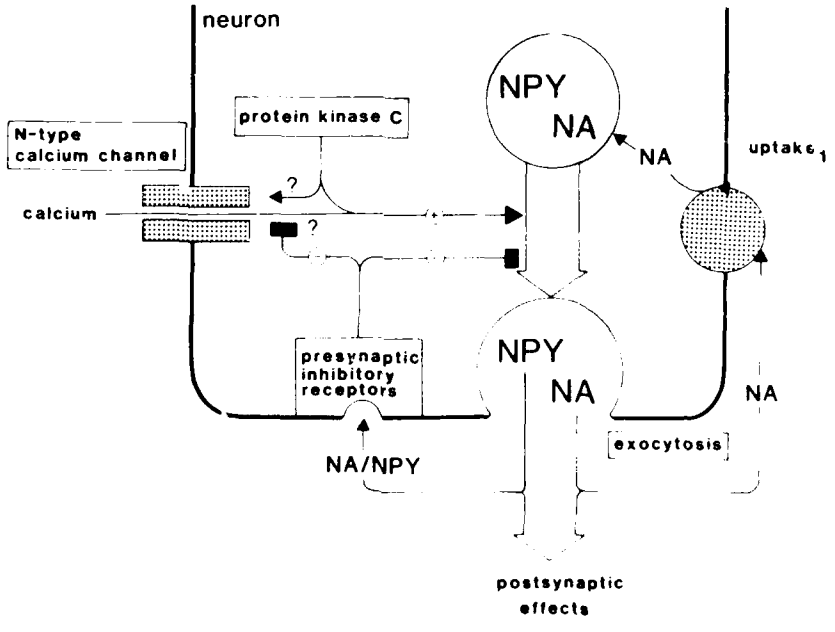

FIGURE 1. Scheme of the regulation of stimulation-evoked corelease of neuropeptide Y (NPY) and noradrenaline (NA) from cardiac sympathetic nerve endings. Briefly, electrical stimulation of postganglionic sympathetic nerve fibres evokes a corelease of both transmitters, that is modulated by presynaptic receptors and requires the presence of extracellular calcium, the influx of calcium through N-type calcium channels and the activation of protein kinase C.

According to an exocytotic release mechanism, the overflow of NPY from the heart evoked by various stimuli, such as nicotine,[6] veratridine,[7] or electrical stimulation[3,5,7] critically depends on the presence of extracellular calcium. Using various ligands for voltage-dependent calcium channels, it can be concluded, that the entry of calcium into the sympathetic nerve ending requires the activation of voltage-dependent N-type but not of L-type calcium channels.[5] Furthermore, activation of protein kinase C by the phorbol ester TPA markedly enhanced, inhibition of the enzyme by polymyxin B completely prevented NPY and noradrenaline overflow. With the exception of studies involving uptake$_1$-blockade, there was a molar ratio of NPY and noradrenaline overflow of approximately 1 : 600.

The regulation of NPY and noradrenaline overflow at the sympathetic nerve ending of the heart is illustrated in FIGURE 1. Whether the regulation of the transmitter release by presynaptic receptors is mediated by modulation of N-type calcium channel gating[8] or by interference with the protein kinase C activity cannot be concluded from our results.

In conclusion, the stimulation-evoked exocytotic corelease of NPY and noradrenaline is characterized by parallel presynaptic regulation, dependence on the presence of extracellular calcium, stimulation of N-type calcium channels, and activation of protein kinase C.

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Effect of Neuropeptide Y on Stimulated Renin Secretion[a]

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INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid peptide with vasoconstrictor properties. This peptide is present in the brain, the adrenal medulla as well as in sympathetic nerve endings. In the kidney, NPY has been found in the fibers innervating the juxtaglomerular apparatus, *i.e.*, in the vascular pole of the glomerulus where renin-secreting cells are located.[1] In the isolated rat kidney, NPY has been shown to inhibit renin release.[2] A suppressing effect of NPY on renin release has also been observed in intact rats with a renin-angiotensin system activated.[3-5] The present study was undertaken to assess in conscious normotensive rats whether exogenous NPY affects renin secretion induced by various stimuli.

METHODS

Male normotensive Wistar rats weighing between 240 and 300 g were used. The animals were cannulated under ether anesthesia with one arterial and two venous catheters. Blood pressure and heart rate were then continuously monitored using a computerized data acquisition system. All experiments were started 90–120 min after the end of surgery, at a time a steady state was reached. A blood sample was obtained at the end of the experiment through the arterial line for measurement of plasma renin activity.

Rats were infused for 45 min with either a nonpressor dose of NPY (0.1 μg/min i.v., Bio Mega, Laval, Canada) or its vehicle. Starting 15 min later, a second infusion was initiated. It consisted either of the beta-adrenoceptor stimulant isoproterenol (10 ng/min i.v.; Winthrop, Basel, Switzerland), or the prostacyclin agonist iloprost (50 ng/min i.v.; Schering, Berlin, Germany).

Results are reported as means ± SEM. Statistical analysis of the results was done using a one-way analysis of variance, followed by a least significant difference test, where appropriate.

RESULTS

NPY had no significant blood pressure effect. Blood pressure remained also unchanged when isoproterenol and iloprost were infused together with NPY. TABLE 1 shows the levels of mean blood pressure measured at the beginning and the end of the experiment in the different groups of rats.

[a]This work was supported by a grant from the Swiss National Foundation.

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Plasma renin activity was not altered by NPY infused alone. However, the peptide significantly attenuated the increase in plasma renin activity caused by both isoproterenol and iloprost (TABLE 1).

DISCUSSION

The first evidence indicating a role for NPY in the regulation of renin release is derived from experiments in which rats with glucocorticoid-induced hypertension were treated with a nonpressor dose of the peptide.[3] Some rats had been subjected to a bilateral adrenalectomy and exhibited very high plasma renin levels. In these rats, NPY allowed plasma renin activity to decrease to levels similar to those measured in control animals with intact adrenals. NPY was also shown to blunt the renin response to ACE inhibition and renal artery clipping.[4,5] The inhibitory effect of NPY on renin secretion was confirmed *in vitro* using kidney slices.[2]

TABLE 1. Mean Blood Pressure (MBP, mmHg) and Plasma Renin Activity (PRA, ng/ml/hr) in the Different Study Groups[a]

					MBP		
Groups	(n)	NPY	Isoproterenol	Iloprost	Before	After	PRA
I	6	−	−	−	119 ± 2	120 ± 2	3.4 ± 0.8
II	7	+	−	−	121 ± 3	126 ± 4	2.5 ± 0.3
III	6	−	+	−	120 ± 2	119 ± 1	16.4 ± 3
IV	7	+	+	−	120 ± 3	120 ± 3	7.21 ± 1.5**
V	8	−	−	+	118 ± 2	116 ± 2	35.0 ± 7
VI	8	+	−	+	120 ± 2	120 ± 2	9.5 ± 2.9**

[a]**$p < 0.01$ vs vehicle of NPY; + test substance infused; − no active treatment.

In the present study, two well established stimuli of renin secretion were infused to conscious rats at doses devoid of systemic blood pressure effect. Both agents markedly stimulated renin secretion. NPY infused at a nonpressor dose had no effect on plasma renin activity in baseline conditions, but markedly suppressed the renin release induced by the two test stimuli.

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Expression of Neuropeptide Y-Like Immunoreactivity Begins after Initiation of Nicotinic Synapse Formation in Developing Sympathetic Ganglia of the Bullfrog Tadpole[a]

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Neuropeptide Y (NPY) in bullfrog sympathetic ganglia is selectively expressed by C cells, a subclass of postganglionic neurons that are vasomotor in function. In addition, bullfrog ganglia contain fast B and slow B neurons that project to nonvascular targets in the skin. These experiments analyze the developmental differentiation of B and C neurons in lumbar paravertebral ganglia 9 and 10 during tadpole stages III to XXV and in juvenile bullfrogs. The process of ganglionic synaptogenesis and the development of axonal conduction velocity were studied by recording postsynaptic compound action potentials from the sciatic plexus. The expression of NPY and of tyrosine hydroxylase (TH), an adrenergic marker, was studied in 10-μ sections of ganglia 6, 9, and 10 using indirect immunofluorescence.

Small postganglionic potentials (3–12 μV in amplitude) corresponding to separate sympathetic B and C systems were present at stage III. The B response was elicited by preganglionic stimulation of the sympathetic chain between ganglia 6 and 7, and the C response was elicited by preganglionic stimulation of spinal nerve 8 central to its ramus communicantes. Synaptic mediation of the postganglionic action potentials was demonstrated by reversible blockade in low calcium, high magnesium Ringer and in nicotine. The combined pre- and postganglionic conduction velocity of the B wave was 0.22 m/s ($n = 2$) and that of the C wave was 0.13 m/s ($n = 2$). Between stages III and XXV, the magnitudes of the responses grew exponentially and reached amplitudes of 200–400 μV. Conduction velocity also increased during development, but at a slower rate. During metamorphic stages (XVII–XXV), the B wave conducted at 0.45 ± 0.07 m/s (mean ± SD, $n = 6$) and the C wave conducted at 0.25 ± 0.04 m/s ($n = 6$). These findings indicate that synapse formation in the B and C systems is selective from the outset and that the number of innervated ganglion cells increases during this period of development. Differentiation of the B wave into fast B and slow B components was first observed in juvenile bullfrogs that had completed metamorphosis between 11 days and 15 weeks earlier. This suggests that the selective myelination of preganglionic B fibers and postganglionic fast B fibers occurs after metamorphosis.

NPY-like immunoreactivity was first observed in a small population (less than 1%) of

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neurons in ganglia 9 and 10 at stage XI. This period marks the beginning of the 'foot' stages of development and coincides with the onset of reflexive movements in the hindlimb. The percentage of cells containing NPY then increases gradually, reaches adult proportions (~55%) by stage XX, and persists there through metamorphosis. In order to determine whether the onset of NPY expression follows a rostro-caudal gradient, counts of NPY positive and negative neurons were made in ganglion 6 and ganglion 9 at stages IX, XV, and XVII. At each stage, the levels of NPY expression were the same in both ganglia. This indicates that the timing of NPY expression is not linked to segmental position. The onset and pattern of NPY expression differed from that of TH. At stage III and all later stages, virtually all ganglion cells were positive for TH. During late metamorphic stages and in juvenile frogs the intensity of TH staining became bimodal; large ganglion cells were intensely positive, and small ganglion cells were weakly positive. Double labeling of sections for TH and NPY revealed that the NPY positive neurons corresponded to the cells that were weakly positive for TH. In six-inch adult bullfrogs, the distinct bimodal pattern of TH staining disappeared.

Together, the results of this study demonstrate that the onset of NPY expression is not tightly linked to the electrophysiological differentiation of B and C cells, ganglionic synaptogenesis, or the adrenergic differentiation of sympathetic neurons. The transient alterations in the intensity of TH staining that occur after the onset of NPY expression indicate that the adrenergic and peptidergic properties of C neurons can be independently regulated. The synchronized onset of NPY expression in different segmental ganglia suggests that NPY expression is not linked to a rostro-caudal gradient of developmental events, but rather to factors that act globally upon developing sympathetic neurons.

Neuropeptide Y Presynaptic Effects in Hippocampal Slice *In Vitro* Are Y_2 Receptor Mediated but Not via Inhibition of Adenylate Cyclase[a]

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Neuropeptide Y (NPY) is found in abundance in the central nervous system of the rat (see this volume). Binding sites for NPY are concentrated in the strata radiatum and oriens of rat hippocampus.[1] NPY potently and reversibly inhibits excitatory synaptic transmission from stratum radiatum to CA1 pyramidal neurons in the *in vitro* hippocampal slice preparation, by directly inhibiting calcium influx into the presynaptic nerve terminal.[2,3] There is evidence for two subtypes of NPY receptor; the receptor mediating presynaptic inhibition in the vas deferens is of the Y_2 subtype.[4,5] Here, we have characterized the NPY receptor mediating presynaptic inhibition in rat hippocampal slice.

Electrophysiological recordings were made from continuously perfused, submerged transverse slices (400–450 μm) of rat hippocampus as previously described.[2,3] The population spike (PS) component of the extracellular field potential evoked by stimulation of stratum radiatum was recorded from the stratum pyramidale of area CA1. Stimulus intensity was adjusted to elicit a PS response of between 50 and 75% of maximum. NPY fragments (generous gifts of Dr. S. St-Pierre and A. Fournier; porcine sequence, C-terminally amidated unless otherwise noted) and drugs were applied via the bath. Comparisons of potency were made at a peptide concentration of 1 μM.

Application of 1 μM NPY caused a potent (89.9 ± 2.8%) inhibition of PS amplitude, which recovered upon prolonged (≥ 1h) washout. The analogues, PYY and human NPY (hNPY) were also very effective at inhibiting synaptic transmission (98.8 ± 1.0% and 91.2 ± 4.9% inhibition, respectively). However, hNPY-COOH, which lacks the C-terminal amide, was without significant effect.

C-terminal fragments of NPY were also effective at inhibiting the PS (FIG. 1). A significant reduction in potency occurred between the 2–36 and 5–36 fragments, between the 11–36 and 13–36 fragments and between the 13–36 and 16–36 fragments. While pNPY 16–36 still inhibited the PS significantly, 25–36 was without effect.

The data indicated a similar structure-activity profile for the hippocampal NPY receptor as that which mediates presynaptic inhibition in vas deferens, the Y_2 receptor.[4,5] Because activation of this receptor inhibits the enzyme adenylate cyclase,[5] we elevated cAMP levels by applying a high concentration of the membrane permeant analogue 8(4-chlorophenylthio) cAMP (CPTcAMP; 100 μM, applied in the presence of the antag-

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onist, 8-p-sulfophenyl theophylline, to prevent cAMP activation of the inhibitory presynaptic adenosine receptor[6]), then tested NPY actions. Under these circumstances, CPT-cAMP elevated synaptic transmission by 206.7 ± 28.8% of control. In the presence of CPTcAMP, NPY inhibited PS amplitudes by 51.8 ± 9.9% of control.

The results show that the presynaptic action of NPY in hippocampus is mediated by a receptor resembling the peripheral Y_2 receptor, but that its action is independent of its effect on adenylate cyclase.

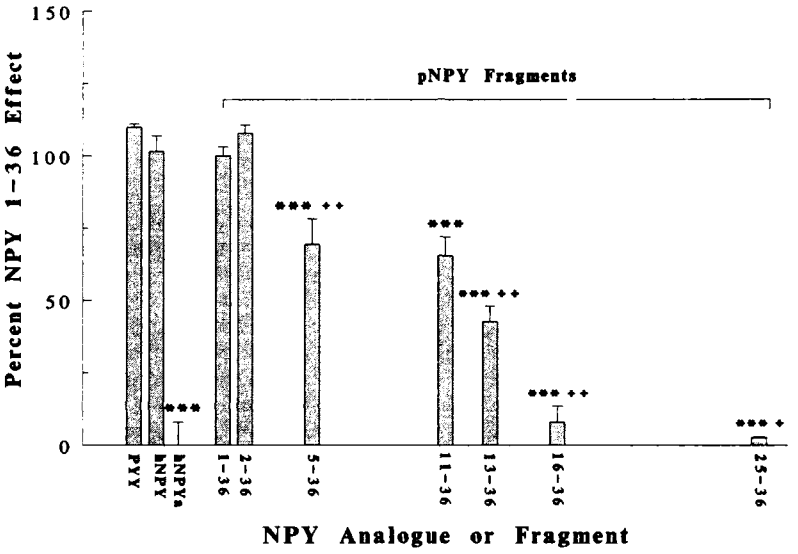

FIGURE 1. Structure-activity relationship of analogues and c-terminal fragments of NPY on the inhibition of excitatory synaptic transmission in rat hippocampal slice, *in vitro*. Comparisons are made at 1 μM peptide concentration. The effect of analogues and fragments are normalized to the effect of porcine NPY1-36-NH_2. Abbreviations: Porcine sequence peptide YY (PYY); human sequence NPY1-36NH_2 (hNPY); human sequence NPY1-36-COOH (hNPYa). All NPY c-terminal fragments tested are porcine sequence. ***p <0.001 compared with pNPY1-36NH_2. + p <0.005, + + p <0.001 compared with previous (longer) fragment.

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Effects of Haloperidol and Cortical Lesions on the Level of Neuropeptide Y mRNA in the Striatum of the Rat

A Study by *In Situ* Hybridization Histochemistry

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High concentrations of neuropeptide Y (NPY) are present in the caudate-putamen (striatum), a structure involved primarily in the control of movement.[1-3] In this area, NPY is colocalized with somatostatin in a population of medium-size interneurons (classified as type IV) which are known to receive direct contacts from the dopaminergic nigro-striatal pathway and from the cerebral cortex.[4,5] Previous immunohistochemical experiments indicate that striatal NPY neurons are regulated by these two main projections to the striatum. In particular, lesions of the dopaminergic nigrostriatal neurons and of the fronto-parietal cortex increase NPY levels in the ventro-medial and dorso-lateral part of the striatum, respectively.[6] In view of the critical role played by nigro-striatal dopaminergic neurons in the extrapyramidal control of movement and the reciprocal influence of NPY and dopamine in the striatum,[7,8] it was of interest to determine whether dopaminergic antagonists differing in their ability to induce motor effects differentially affected striatal NPY neurons. In the present study, we have measured by *in situ* hybridization histochemistry the level of messenger RNA (mRNA) encoding NPY in the rat striatum, nucleus accumbens and frontal (motor) cortex after treatment with either vehicle (0.5 ml Tween), haloperidol (1 mg/kg, 28 days) an antipsychotic which produces motor side effects, or clozapine (20 mg/kg, 28 days) which does not. We have also examined NPY mRNA expression in the striatum 5 and 21 days after extensive fronto-parietal lesions by thermocoagulation. The brains were frozen and frontal sections (10 μm) were cut on a cryostat between A 9.2 and A 10.6 according to the atlas of Paxinos and Watson (1986). The NPY mRNA was detected with an antisense 35S-labeled cRNA probe which was transcribed from a cDNA clone provided by Dr. S. Sabol (NIH). Topographical maps of the labeled cells were drawn using dark-field microscopy. The number of labeled cells was measured in each structure considered. To measure the intensity of labeling per cell, the digitized image of each cell, identified on the topographical map, was placed in the middle of the video screen within a circle of fixed surface area, and the area covered by silver grains was measured with a Morphon image analysis system. The validity of these measurements, expressed as the number of pixels per cell, was confirmed by comparing the values obtained by visual grain counting and computer-assisted image analysis in a sample of cells from each structure examined and from each experimental group (correlation coefficient >0.95). Differences in the number of labeled cells and in the number of pixels/cell among structures of experimental groups were assessed using the Mann-

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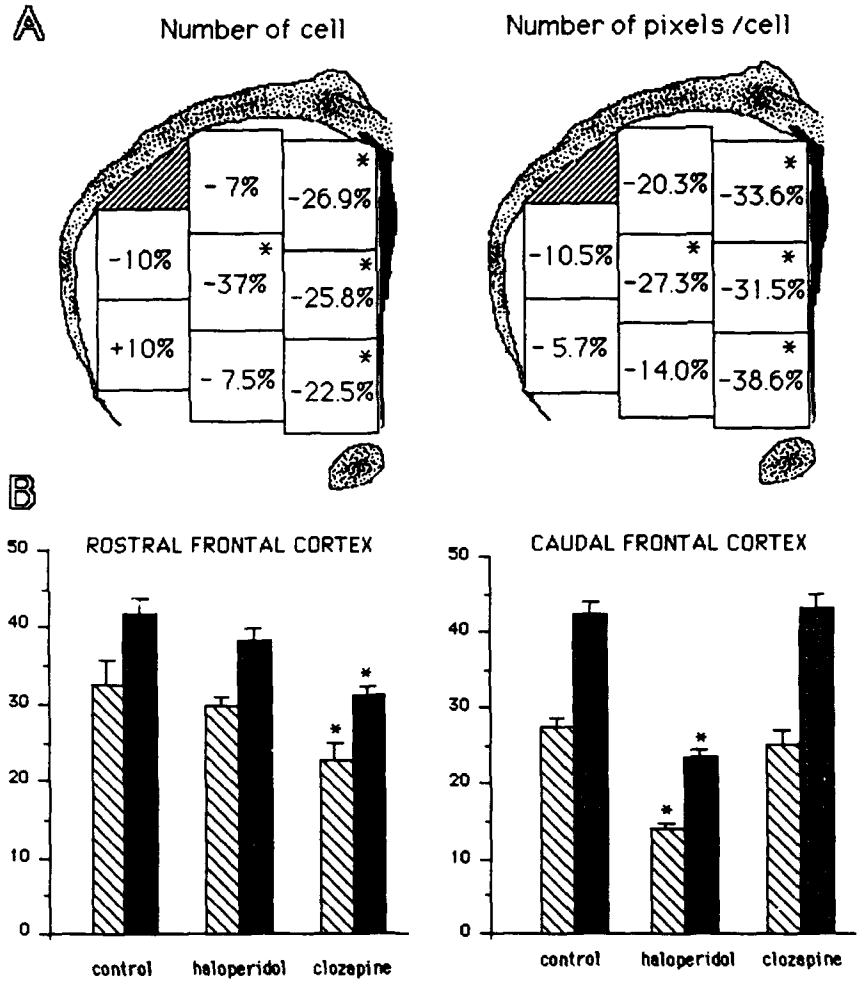

FIGURE 1. **(A)** Topographical analysis of the intensity of labeling (pixels) and number of labeled cells in the rat striatum after chronic haloperidol treatment (1mg/kg; 28 days). The mean ± SEM of the average number of pixels/cell and labeled cells in 5 animals were different from control in areas labeled with *($p < 0.01$ with Mann Whitney U test). Data show per cent changes calculated in comparison with values of corresponding areas in control rats. **(B)** Effects of chronic treatment with haloperidol and clozapine on the level of neuropeptide Y mRNA in the rostral and caudal frontal cortex. Data are the mean ± SEM ($n = 5$) of the average labeling measured in at least 3 sections per animal and per level. *Light bars:* number of cells and *dark bar:* number of pixels/cell after haloperidol (1 mg/kg; 28 days) and clozapine (20 mg/kg; 28days) treatment. *$p < 0.005$ as compared to control values, with the Mann-Whitney U statistic.

Whitney statistic. In addition, because no differences were found within each group (Dunnett test), values from all animals of each group were pooled and the frequency distributions of the number of pixels/cell were plotted for each region and each experimental group, and compared using the two-tailed Kolmogorov-Smirnov sample test.[9]

In control rats, a higher density of NPY neurons was found in the nucleus accumbens (14.86 ± 0.80 cells/mm^2) than in the striatum (5.78 ± 0.19) and the frontal cortex (6.82 ± 0.64), but the level of labeling per cell was higher in the frontal cortex (41.70 ± 1.95 pixels/cell) than in the striatum (29.67 ± 0.70) and the nucleus accumbens (23.83 ± 0.53). The number of cells containing NPY mRNA and the intensity of labeling per individual neurons were not significantly different in all striatal subregions.

Chronic treatment with haloperidol, but not with clozapine decreased NPY mRNA expression in the nucleus accumbens (-24.1%), ventro-median striatum and caudal frontal cortex (FIG. 1). Clozapine treatment decreased the levels of NPY mRNA only in the rostral part of the frontal cortex (FIG. 1). The levels of NPY mRNA were increased in the dorso-lateral part of the striatum 5 and 21 days after unilateral cortical lesion by thermocoagulation (FIG. 2).

The differences of NPY mRNA labeling observed in the striatum, nucleus accumbens and frontal cortex of untreated rats suggest that NPY neurons in these three structures are differentially regulated. The differences in the effects of haloperidol, a typical neuroleptic, and clozapine, an atypical antipsychotic drug, on NPY mRNA expression in the striatum, nucleus accumbens, and frontal cortex may be related to a role of NPY interneurons in the regulation of movement. In the striatum, similar results have been found for the mRNA encoding somatotastin, a neuropeptide present in the same neurons as NPY in this region. However, different effects were observed in the nucleus accumbens after the same treatments[9] suggesting that somatostatin and NPY gene expression are differentially regulated in this area. The results suggest that NPY gene expression in the rat striatum is under the influence of both cortico- and nigro-striatal afferent pathways. The changes in mRNA levels are likely involved in the regulation of NPY production previously reported with immunohistochemical methods.

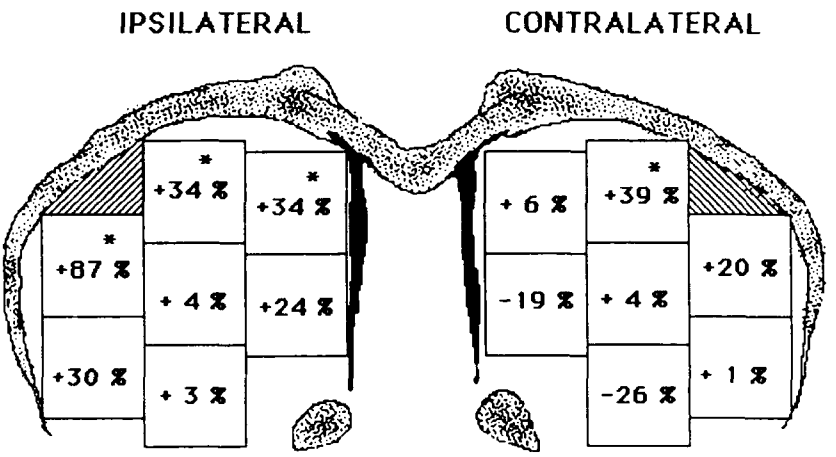

FIGURE 2. Effects of fronto-parietal lesions on neuropeptide Y mRNA expression in the rat striatum. Results are expressed as the per cent change induced 5 days after the unilateral cortical lesion in the mean number of striatal neurons labeled for neuropeptide Y mRNA, counted inside each striatal area and compared to the corresponding control values. *$p < 0.01$ using the Mann-Whitney U test.

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Regulation of Neuropeptide Y in Neural and Cardiac Culture

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We have examined the regulation of Neuropeptide Y (NPY) mRNA levels, peptide synthetic rate and peptide content in primary cell cultures from rat superior cervical ganglion (SCG)[1,2] and atrium.[3] SCG and atrial cultures were established from 1–2-day-old rats and cultures were maintained in serum-free medium for 2–3 weeks. NPY was stable in spent medium for at least 48 hours. Since NPY is colocalized with norepinephrine (NE) in SCG,[1] we have compared the regulation of NPY and NE synthesis[1] and NPY and tyrosine hydroxylase (TH) mRNA[4] in these neurons after treatment of cultures with several adrenergic agents. These data show that several important regulatory effects can be seen in the primary neuronal cultures (TABLE 1). First, despite the marked changes in NE synthesis and accumulation, the cultures never show any change in NPY synthesis and accumulation in response to a variety of adrenergic drugs. Second, the acute and chronic effects of these drugs on NE synthesis clearly differed. In particular the chronic effects of desipramine and clonidine were significant, but no acute effects on NE synthesis were seen.

Factors which regulate signal transduction pathways frequently after peptide synthesis and secretion. As shown in TABLE 2, treatment with cAMP increased NPY and TH mRNA levels without changing NPY or NE synthesis and accumulation. PMA reduced NPY and NE synthesis and accumulation, but had little effect on NPY or TH mRNA levels. In general chronic treatment of SCG cultures with cAMP, PMA and dexamethasone regulated NPY and NE synthetic rates and NPY and TH mRNA levels in parallel. Since the changes in synthetic rate (NE and NPY) and mRNA levels (TH and NPY) were not always in agreement, the mechanism of peptide and transmitter regulation probably involves interesting posttranscriptional regulatory events.

There is mounting evidence that NPY plays an important role in the mammalian cardiovascular system.[5] We investigated the effect of cAMP, PMA and Dex on NPY synthesis in primary atrial cultures. Treatment with cAMP and dexamethasone markedly reduced NPY content in spent medium and reduced NPY mRNA levels in cell extracts. In contrast treatment with PMA increased NPY content in spent medium and increased NPY mRNA in cell extracts. These data demonstrate that the regulation of NPY in neuronal and atrial culture is tissue-specific.

TABLE 1. Effect of Adrenergic Drugs on Neurotransmitter Synthetic Rates[a]

	Norepinephrine			
	Acute	Chronic	NPY	Action on Catecholamines
Reserpine	down	down	nc	block uptake into granules
Tyramine	down	down	nc	fill granules with octopamine
Desipramine	nc	down	nc	block presynaptic uptake of NE
Clonidine	nc	down	nc	α_2 agonist
Yohimbine	nc	nc	nc	α_2 antagonist

[a]nc = no change.

TABLE 2 SCG Cultures: Parallel Effects on NE and NPY[a]

Treatment	Synthesis NPY,NE	mRNA NPY,TH
cAMP	nc	166%
PMA	40%	nc
Dexamethasone	133%	nc

[a]nc = no change.

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Clinical Evaluation of Neuropeptide Y as a Plasma Marker of Tumors Derived from Neural Crest

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Neuropeptide Y (NPY) is a 36 amino acid peptide, which was originally isolated from the porcine brain.[1] NPY is known to exist in the central nervous system, sympathetic ganglia, peripheral sympathetic nerves and adrenal medulla in high concentrations.[2-4] High concentrations of NPY in plasma and tumor tissues of pheochromocytoma (Pheo) and ganglioneuroblastoma patients had already been reported.[5,6]

To evaluate NPY as a plasma marker of tumors, we measured plasma and tissue immunoreactive (IR)-NPY levels by radioimmunoassay (RIA) as described elsewhere.[7,8] The serial dilution curves of the extracts of both the normal mixed plasma and the plasma of a Pheo and a neuroblastoma were parallel to the standard dilution curve (FIG. 1). Plasma IR-NPY levels in 17 of 21 Pheo were higher than 290 pg/ml, which is the upper limit of the plasma IR-NPY levels in normal adult subjects. Plasma IR-NPY levels in 19 of 21 ganglioneuroblastomas or neuroblastomas (NB-group) were higher than 350 pg/ml, which is the upper limit of the plasma IR-NPY levels in normal children. Plasma IR-NPY levels in 2 of 4 cases of rhabdomyosarcoma were very high (1611 and 3632 pg/ml) (TABLE 1). High levels of IR-NPY were also found in tumor tissues of Pheo (0.025–95.3 μg/g wet tissue, n = 11) and NB-group (0.07–5.33 μg/g wet tissue, n = 5). An analysis using reverse phase high performance liquid chromatography revealed that IR-NPY in plasma extracts and tumor tissue extracts of Pheo and NB-group was eluted in an identical position to synthetic human NPY in most cases (data not shown). Notably, plasma IR-NPY levels in patients with chronic renal failure were also high suggesting that the renal clearance of NPY in these patients is decreased.

These findings indicate that, in case of normal renal function, NPY is a useful plasma marker of tumors originating from the neural crest. Further investigation is required as to the mechanism of the NPY-gene expression in these tumors.

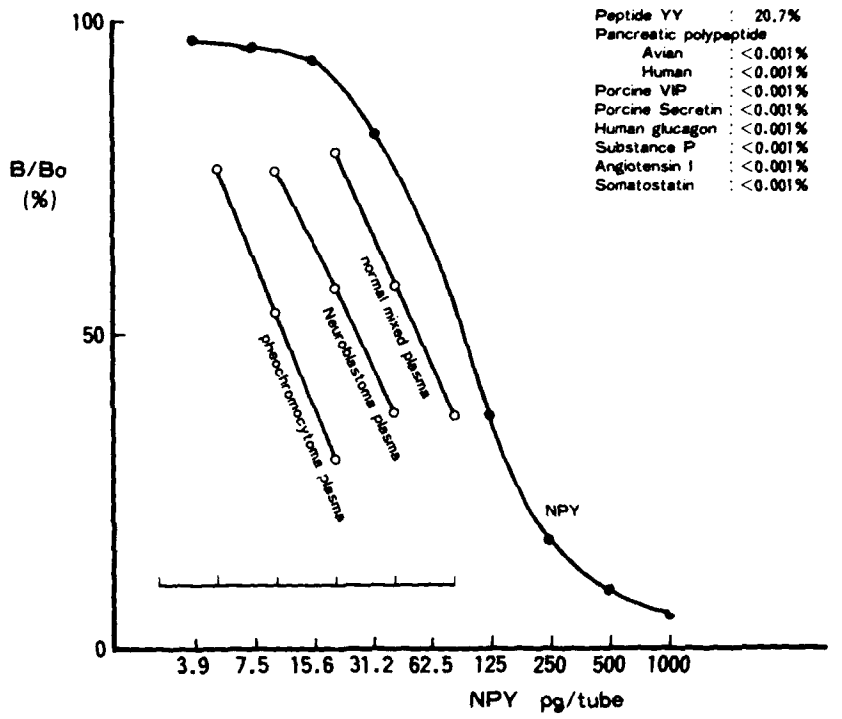

FIGURE 1. A standard curve of NPY (●), serial two-fold dilution curves (○) of a normal mixed plasma extract, a pheochromocytoma plasma extract, a neuroblastoma plasma extract and cross reactivities.

TABLE 1. Plasma IR-NPY Concentrations[a] in Various Diseases

	N	IR-NPY (pg/ml)
Normal subjects	21	151 ± 29
Essential hypertension	35	175 ± 48
Pheochromocytoma	21	1636 ± 3902
Neuroblastoma group	21	1748 ± 1827
Rhabdomyosarcoma	4	1611 ± 3632
Endocrine diseases	39	138 ± 31
Brain tumors	4	145 ± 30
Carcinomas	11	199 ± 60
Chronic renal failure		
without hemodialysis	8	330 ± 63
with hemodialysis	10	374 ± 80

[a]Values are represented as the means ± SD.

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Neuropeptide Y Regulation of LHRH Release in Ewe Median Eminence

Immunocytochemistry, Tissue Content, and *In Vivo* Analysis[a]

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Luteinizing hormone (LH)-releasing hormone (LHRH) neurons are the main element of the hypothalamic hypophysiotropic reproductive network. While synchronization within this network might be achieved through synapses among LHRH neurons, modulation of its activity preceding the preovulatory surge of LHRH is probably related to its neurotransmitter and/or peptidergic synaptic inputs. Stimulatory and inhibitory inputs exist at the level of LHRH cell bodies in the medial preoptic nucleus and medial basal hypothalamus (MBH), and at the level of their terminals in the median eminence (ME). The preovulatory surge of LHRH is probably related to a temporal predominance of facilitatory over inhibitory inputs to this LHRH neuronal network.

In rats and hamsters, NPY-containing perikarya are located in the MBH and NPY-containing axons converge toward the ME.[1,2] Furthermore, both a stimulatory and an inhibitory role of NPY on LHRH release has been reported in rats and rabbits.[3-7] For example, the high circulating levels of serum LH observed after castration are dramatically decreased by NPY administered in the third ventricle of male and female rats.[3-6] NPY administration also decreases pulsatile LH secretion in ovariectomized rats.[4] Third ventricular administration of NPY also induces a prolonged inhibition of LH in intact male rats,[6] as does NPY perifusion of the MBH in ovariectomized females and intact male rabbits.[7] In contrast, the latter procedure increases *in vivo* LHRH release in intact female rabbits.[7]

In the ewe, *in vivo* ME-NPY release, assessed by push-pull cannula (PPC) sampling, is higher in estrous than during the luteal or anestrous period (FIG. 1). Perfusion of NPY (5 μg/200 μl perfusate) through the PPC in the posterior-lateral ME increased *in vivo* LHRH release in estrous but not in luteal or anestrous ewes. No effect was observed when NPY was perfused in the anterior-lateral ME. On the other hand, our immunocytochem-

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ical studies indicate that NPY-containing perikarya are located in the arcuate (arn) area, while NPY-containing axons converge toward the ME and descend into the infundibulum (inf) of the ewe (results not shown). Finally, discrete microdissection of these areas followed by specific RIA indicate that NPY content is higher in the ME (stalk and inf/arn) than in the lateral peri-arn (105 ± 20 vs 30 ± 6 pg/μg protein, mean ± SEM, n = 25). Similarly, NPY content in the ME (stalk and inf/arn) is highest in late luteal and lowest in mid-luteal ewes (132 ± 35 vs 82 ± 8).

A stimulatory and possibly synaptic NPY-LHRH interaction, restricted to the posterior-lateral ME and probably modulated by estradiol, might trigger the natural preovulatory surge of LHRH in the ewe. This suggestion is based on our immunocytochemical and *in vivo* NPY and LHRH data, specifically: a) the similar projection areas of NPY- and

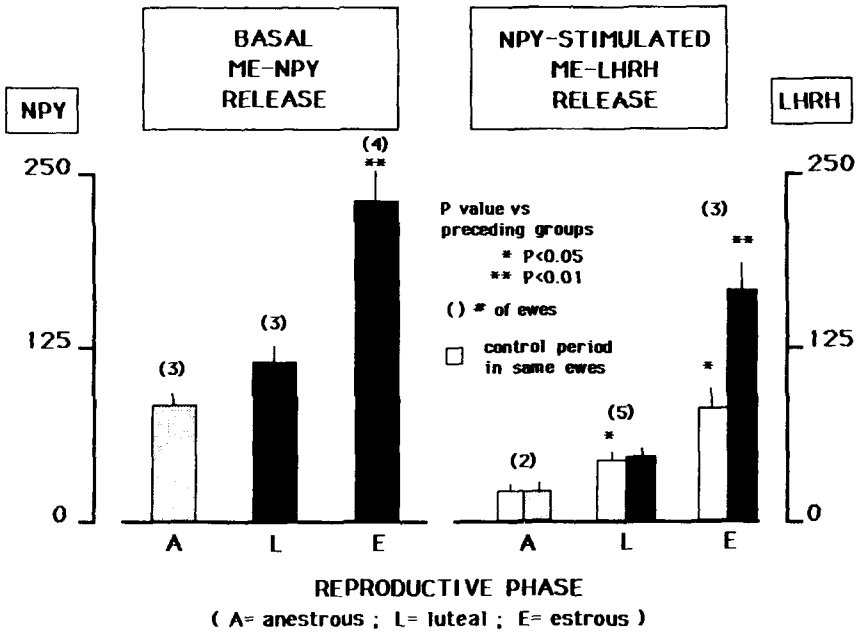

FIGURE 1. Median eminence (ME) *in vivo* release assessed by push-pull cannula (PPC) sampling (pg/200 μl PPC perfusate/20 min during 120 min).

LHRH-containing axons toward the ME provide opportunities for potential synaptic interactions; b) NPY increases *in vivo* ME-LHRH release only when serum estradiol (E2) is high and serum progesterone (P4) is low, but is ineffective when E2 is low and P4 is high, or when serum levels of both steroids are low (anestrus); c) *in vivo* basal ME-NPY release is higher in estrous than in luteal ewes; and d) ME content of NPY is highest at the end of the luteal period of cycling ewes.

That E2 might be involved in the LHRH response to NPY is also supported by results from the rat.[8-12] For example, NPY-induced release of LHRH from MBH-ME fragments is greatly enhanced by E2 but not by P4.[8-10] Pharmacological doses of P4 actually decrease the E2-facilitated NPY-induced release of ME-LHRH.[9] In addition, the effects of NPY on LHRH have also been associated with alterations in LHRH content in various

hypothalamic areas in the rat. For example, E2 decreases NPY content, while P4 induced only a transient decrease and a subsequent rise of NPY content in the interstitial nucleus of the stria terminalis, the arcuate, and the preoptic area.[12]

Thus, a stimulatory and possibly synaptic NPY-LHRH interaction, restricted to the posterior-lateral ME and probably modulated by estradiol, might trigger the natural preovulatory surge of LHRH in the ewe.

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A Role for Neuropeptide Y in the Mediation of Glucocorticoid-Induced Changes in Hypothalamic Function[a]

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Immunocytochemical studies have shown abundant NPY nerve terminals in a number of hypothalamic areas and nuclei.[1,2] The two main sources of this NPY innervation are projections from brainstem catecholaminergic cell groups[1] and a dense population of neuronal cell bodies located in the arcuate nucleus.[2] In both the brainstem and the hypothalamus, the majority of NPY perikarya contain a high density of nuclear glucocorticoid receptors (GR) indicating a direct sensitivity to changes in adrenocortical function.[3] This may be of particular relevance to the regulation of the hypothalamo-pituitary-adrenal axis as corticotropin releasing hormone neurones in the paraventricular nucleus (PVN) receive a dense NPY innervation from both the brainstem and arcuate nucleus (ARC).[1,2] To evaluate the influence of glucocorticoids on the function of NPY neuronal systems in the hypothalamus, we have performed a number of different investigations,[4,5] whose findings are briefly summarised here.

Primary cultures of hypothalamic neurones prepared from neonatal rats were maintained for one week in serum free medium containing different concentrations of dexamethasone. Intracellular levels of NPY demonstrated concentration-dependent increases, with the content being 2–3-fold higher than control after treatment with dexamethasone at 10^{-8} and 10^{-7} (FIG. 1).[4] A positive effect of glucocorticoids was also observed when female Wistar rats (200 g) were administered 100 μg dexamethasone *i.p.* per day for 5 days, tissue levels of NPY being increased by 117% in the medial basal hypothalamus ($p < 0.002$).[4]

However, a study in male Wistar rats designed to further clarify the effect of glucocorticoid excess produced a less marked response. Microdissection of the ARC from rats sacrificed 10 days after receiving a depot preparation of betamethasone (BM, 1.5 mg) did not show significant increases in NPY (BM 55.1 ± 1.9 fmol/μg protein, control 49.8 ± 4.6). But, in the same study, adrenalectomy (ADX) performed 12 days before sacrifice was found to significantly reduce levels (37.8 ± 2.7, $p < 0.02$). A similar pattern of NPY values was observed in the PVN of these animals (control 37.4 ± 3.1, BM 36.8 ± 1.8, ADX 28.8 ± 3.3 $p < 0.05$ v. control). In contrast, norepinephrine (NE) levels in the PVN were not significantly altered by ADX but showed a significant depression after BM administration (NE fmol/μg protein; control 753 ± 48, BM 568 ± 58 $p < 0.05$, ADX 642 ± 66).

[a]This work was supported by the Swiss National Research Foundation (3.091.087).

Microdissection of 11 nuclei and areas from 19 human hypothalami collected at postmortem showed a wide range of NPY values in the infundibular nucleus as well as in other hypothalamic nuclei.[5] Retrospective analysis of glucocorticoid administration showed that this was not the cause of the differences. However, analysis of the various causes of death revealed that NPY levels were significantly higher in the infundibular, ventromedial (VM) and paraventricular nuclei of subjects suffering from chronic respiratory failure (>10 days), a condition associated with sustained distress (FIG. 2).[5] Moreover, NPY values in the infundibular nucleus demonstrated a good correlation with values for the VM ($r = 0.89$) and PVN ($r = 0.84$). Whether, as in the rat,[2] this results from neuronal projections from the infundibular awaits further investigation.

From these different studies, it may be concluded that the function of hypothalamic NPY neurones is not simply related to circulating glucocorticoid levels but is also dependent on the simultaneous activation of other interconnecting neuronal pathways occurring during situations of chronic stress. For example, Sahu and colleagues have reported increases in the NPY levels of the ARC and PVN when rats have been deprived of food for 3 to 4 days.[6] Although it was concluded that this was the result of altered feeding behaviour,[6] it cannot be excluded that a stress of this duration would not also produce a similar change in NPY levels.

Microinjections of NPY into the third ventricle or directly into specific hypothalamic areas have been reported to produce a number of effects ascribed to specific functions within the hypothalamus.[7] These include phase shifting of circadian rhythms, stimulation of ACTH secretion, increasing food intake, alterations of blood pressure, and both inhibition and stimulation of LH secretion depending on the hormonal background at the time of NPY administration. Therefore, the interaction of glucocorticoids with hypothalamic GR-containing NPY neurones could directly modulate the neural circuitry responsible for the physiological regulation of these different neuroendocrine responses.

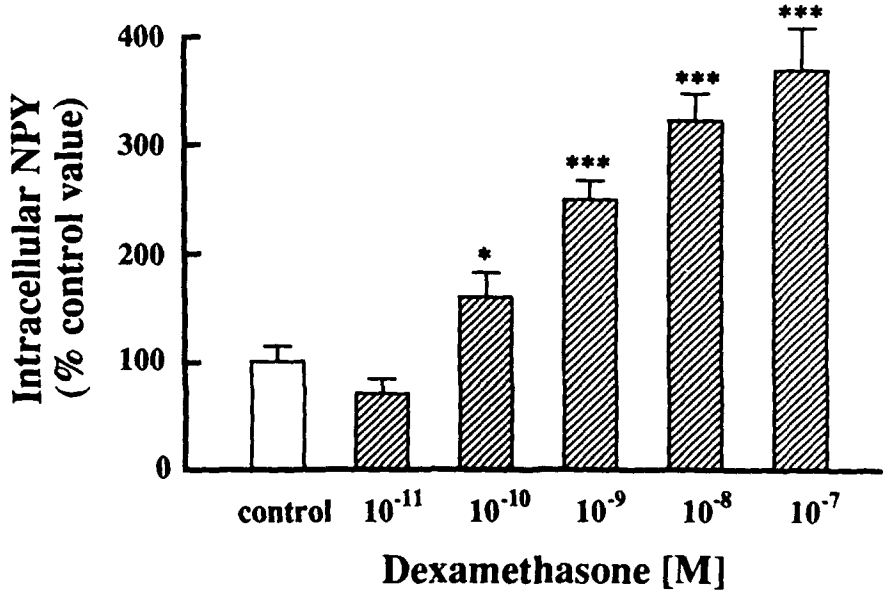

FIGURE 1. Effect of different dexamethasone concentrations on the NPY content of cultured rat hypothalamic neurones maintained for one week in serum free medium (*$p < 0.05$, ***$p < 0.001$).

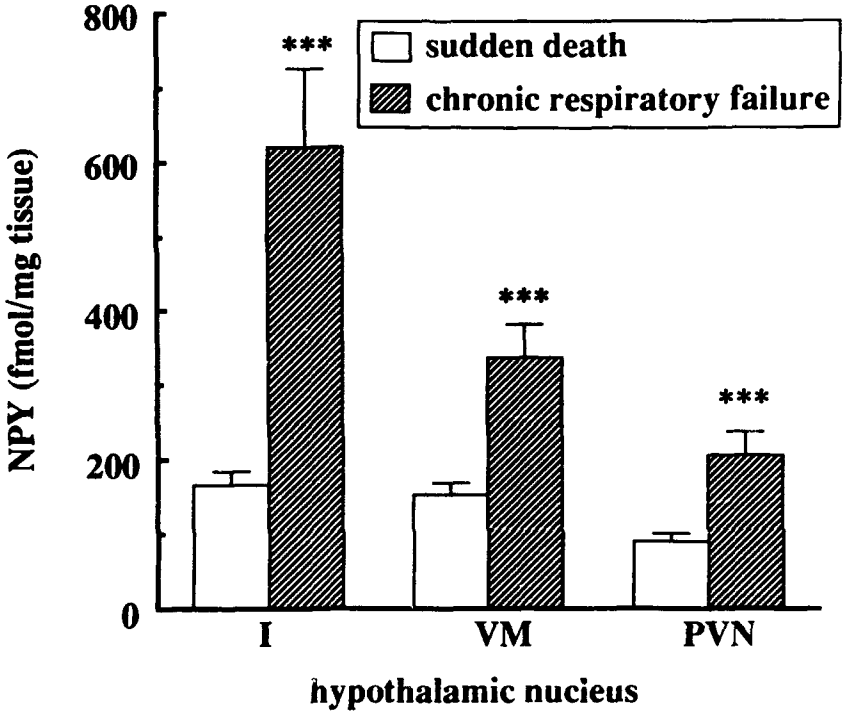

FIGURE 2. Comparison of tissue levels of NPY in the infundibular (I), ventromedial (VM) and paraventricular nuclei (PV) from patients dying suddenly (n = 11) or after a period of chronic respiratory failure of severe dyspnea (n = 8, ***p <0.001).[5] (From Corder *et al.*[5] Reprinted by permission from *Neuroendocrinology*.)

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Comparison of Hypotension-Induced Neuropeptide Y Release in Rats Subjected to Hemorrhage, Endotoxemia, and Infusions of Vasodepressor Agents[a]

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Consistent with its potent vasoconstrictor properties and its ability to enhance pressor responses of adrenergic agonists,[1] early investigations of the peripheral distribution of NPY identified perivascular sympathetic neurones as one of the major sources of immunoreactivity.[2] In addition, NPY was localised to a subpopulation of adrenal medullary cells and to intrinsic nonadrenergic neurones of the heart and gastrointestinal tract.[2] Subsequent studies have attempted to define the physiological stimuli evoking the release of NPY-like immunoreactivity into the circulation from either the adrenal medulla or neuronal sources.[3] Because of the vasoconstrictor action of NPY, it is of particular physiological interest when release occurs during activation of the sympathetic nervous system with simultaneous vasoconstriction of peripheral vascular beds, such as during hemorrhagic hypotension.[4,5] In contrast, no change in plasma levels of NPY was observed in a canine model of endotoxin-induced shock despite large increases in circulating catecholamine concentrations.[6] Experimental models of septic shock as well as the clinical condition are characterised by a reduced responsiveness to pressor amines.[7] If NPY release from perivascular nerves regulates the response of vascular smooth muscle to norepinephrine, inadequate or decreased release of NPY could in part explain the reduced efficacy of vasoconstrictor agents during endotoxemia. The studies summarised below have been performed to further evaluate the relationship between hypotension and NPY release.

Earlier observations demonstrated the important influence of psychological stress on plasma levels of NPY in the rat.[3] Hence to avoid the effect of psychogenic stimuli, the different manipulations leading to hypotension were carried out in anesthetised rats. The first of these studies compared circulating levels of NPY and catecholamines following 30 and 60 min of either hemorrhage or endotoxin-induced hypotension (FIG. 1). In agreement with the results of other groups suggesting a generalised activation of perivascular sympathetic neurones,[4,5] hemorrhage produced marked increases in plasma NPY levels (2.5- and 3.5-fold increases at 30 and 60 min respectively). In contrast, NPY values during endotoxemia were not significantly different from control values despite comparable decreases in MABP to the hemorrhage group. Plasma catecholamine levels were in-

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creased by both types of shock (FIG. 1). The very high levels of E particularly after endotoxin administration suggest that a large component of the increase in NE is also of adrenal medullary rather than of neuronal origin.

Previously it was found that NPY infusion can restore the pressor response to norepinephrine in nonhypotensive endotoxemia.[7] The very high circulating concentrations of catecholamines but apparent absence of increased NPY release after endotoxin administration, even with marked hypotension, led us to evaluate whether infusing NPY reversed the fall in MABP during endotoxin shock. Endotoxin was administered to anesthetised rats as a bolus (2.5 mg/100 g) at 0 min, and 10 min later a 1 h *i.v.* infusion of NPY (10 pmol/100 g/min) or vehicle was started. Although administration of this dose of NPY was without effect on MABP in control rats, it produced a partial reversal of the fall in MABP in endotoxin-treated rats to -13 ± 4 and -14 ± 6 mmHg after 30 and 60 min infusion ($p < 0.01$), compared to -34 ± 5 and -35 ± 6 in rats receiving vehicle. Increasing the dose of NPY to 30 pmol/100 g/min had no greater effect. Infusion of norepinephrine (30 ng/100 g/min), instead of NPY, had no effect on the time course of changes in MABP during endotoxin shock even though it raised MABP by 30–40 mmHg in control animals.

To investigate the apparent suppressed NPY release during endotoxemia, relative to the fall in blood pressure, we compared the effect of infusing different depressor agents over a 30-min period to anesthetised rats (FIG. 2). In this study, levels of NPY were not significantly different from control values after CGRP or nitroprusside infusions. In contrast, infusions of salbutamol and VIP significantly increased plasma concentrations of NPY ($p < 0.05$ and < 0.01).

In summary, endotoxin-induced hypotension in the rat differs from hemorrhagic shock in that NPY release is not markedly increased. However, reduced responsiveness to endogenous and exogenous[7] catecholamines during endotoxemia can be partially overcome by NPY infusion. The results obtained after infusion of different vasodilators

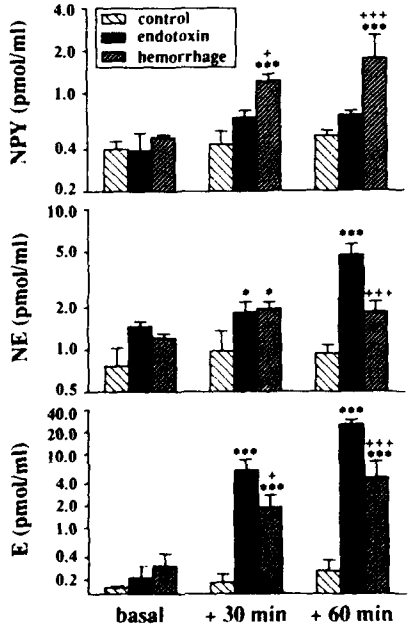

FIGURE 1. Comparison of changes in plasma levels of NPY, norepinephrine (NE) and epinephrine (E) after endotoxin (n = 6, bolus at 0 min of 1 mg/100 g *E. coli* lipopolysaccharide 0111:B4 *Difco Laboratories*, followed by infusion of 10 μg/100 g/min) or 25% hemorrhage (n = 5), compared with control rats (n = 5). Male Wistar rats (290–320 g) were anesthetised by *i.p.* injection of 4 mg/100 g pentobarbitone and 5 mg/100 g ketamine. Mean arterial blood pressure (MABP, mmHg) at 30 and 60 min was decreased by 30 ± 5 and 33 ± 6 during endotoxin-induced shock; and by 43 ± 5 and 45 ± 6 during hemorrhagic hypotension. NPY was measured by direct radioimmunoassay. Statistical analysis was performed by ANOVA; $*p < 0.05$, $***p < 0.001$ v. corresponding control sample; $+p < 0.05$, $+++p < 0.001$ v. corresponding endotoxin sample.

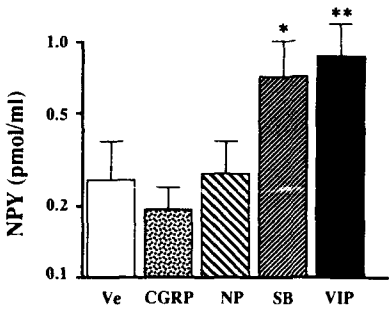

FIGURE 2. Effect of hypotension induced by different vasodilator agents on plasma levels of NPY measured by radioimmunoassay. Male Wistar rats (280–320 g) were anesthetised by *i.p.* injection of 10 mg/100 g ketamine and 0.5 mg/100 g midazolam, and infused an *i.v.* maintenance mixture of 4.8 mg/100 g/h ketamine and 0.2 mg/100 g/h midazolam. Vehicle (Ve, 1% human serum albumin in 0.9% saline, n = 10, calcitonin gene-related peptide (CGRP, 25 pmol/100 g/min, n = 10), sodium nitroprusside (NP, 15 μg/100 g/min, n = 10), salbutamol (SB, 0.2 μg/100 g/min, n = 8) and vasoactive intestinal peptide (VIP, 25 pmol/100 g/min, n = 10) were infused for 30 min. Highly significant falls in MABP ($p < 0.001$) of 34 ± 2, 40 ± 5, 22 ± 4 and 48 ± 4 mmHg for CGRP, NP, SB and VIP respectively were observed after 5 min infusion. The depressor effects were sustained throughout the infusion period with corresponding reductions of 44 ± 2, 50 ± 3, 37 ± 7 and 45 ± 5 mmHg being recorded at +30 min (*$p < 0.05$, **$p < 0.01$).

demonstrate the complexity of the regulation of NPY release in rats subjected to different manipulations leading to hypotension.

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Food Deprivation Modulates Neuropeptide Y Gene Expression in the Murine Hypothalamus

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The behavioral effects of central administration of Neuropeptide Y (NPY) suggest that it participates in the control of food intake. Increased amounts of immunoreactive NPY have been found in the hypothalamus of two models of caloric deficit: the food-deprived rat[1] and the rat with streptozotocin-induced diabetes.[2] Since NPY is synthesized in the arcuate nucleus of the hypothalamus,[3] as well as in noradrenergic neurons of the brainstem which project to the hypothalamus,[4] the source of the elevated NPY is uncertain. We reasoned that detection of fasting-induced changes in NPY messenger RNA would identify the site of increased peptide synthesis. Quantitative Northern blot analysis of the hypothalami and brainstems of *ad libitum*-fed and 24-hour-fasted CF1 mice indicates that only the hypothalamus exhibits fasting-induced increases in NPY messenger RNA levels.

Our results show that NPY neurons in the hypothalamus of mice are sensitive to food deprivation. Other studies have shown that NPY can increase food intake[5] as well as decrease energy expenditure.[6] Thus, hypothalamic NPY neurons may mediate some aspects of the physiological and behavioral changes associated with fasting.

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Steroid Hormone Regulation of Brain Neuropeptide Y mRNA Levels

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Gonadal steroid regulation of the central nervous system (CNS) and pituitary function is one of the most thoroughly described endocrine feedback systems. Luteinizing hormone (LH), released from the anterior pituitary, acts directly on the ovaries to cause ovulation. The preovulatory LH surge is regulated by complex interactions between several neuroendocrine factors and the proper steroid hormone environment, *i.e.*, estradiol (E_2) and progesterone (P_4). Possible sites of action for E_2 and P_4 in regulating serum LH levels include the anterior pituitary (the source of LH) and the preoptic area-hypothalamus (the source of LHRH). For example, there is substantial evidence that E_2 and P_4 affect the LHRH system; however, there are no steroid receptors within LHRH-containing neurons. Thus, steroid hormones are likely to act via a system, or systems, impinging upon LHRH neurons, *e.g.*, NPY.[1] High concentrations of NPY-containing cell bodies are found in the arcuate nucleus of the hypothalamus and the A1 region of medulla,[2] two brain areas that are known to project to the preoptic area, to modify LHRH release, and to contain steroid receptors. NPY does alter serum LH levels[3,4] and *in vitro* release of LHRH[5] with the specific effect dependent upon the steroid hormone environment. Therefore, NPY neuronal pathways are likely candidates for gonadal steroid hormone regulation of CNS gene expression.

Initial nuclease protection experiments examined the effect of ovariectomy (OVX) on hypothalamic preproNPY mRNA levels (TABLE 1) indicating that there is no difference in preproNPY mRNA levels with time postOVX from 7 to 28 days. A second set of experiments examined the effect of steroid hormones on hypothalamic preproNPY mRNA (TABLE 1) showing that E_2 treatment significantly decreased preproNPY mRNA relative to the OVX group ($p < 0.05$) and that 6 h of P_4 treatment are required to significantly increase preproNPY mRNA levels relative to the E_2-treated group ($p < 0.05$). The cellular localization of preproNPY mRNA was assessed in the arcuate nucleus of E_2- and E_2/P_4-treated OVX rats (TABLE 2) confirming P_4 stimulation of hypothalamic preproNPY expression.

These results demonstrate that hypothalamic preproNPY mRNA is regulated by physiological steroid levels within a time frame when these steroids are inducing both LHRH release and an LH surge. Thus, the multiple steroid effects on the LHRH system may be mediated by steroid effects on a CNS NPY system. Future experiments will examine whether NPY-containing neurons are capable of responding directly to E_2 and P_4.

TABLE 1. Nuclease Protection Analysis of Hypothalamic PreproNPY mRNA Levels from OVX and Steroid-Treated OVX Rats[a]

Experimental System	Experimental Group	PreproNPY mRNA
Ovariectomy	7 day	1.20 ± 0.28
	10 day	0.94 ± 0.21
	13 day	0.99 ± 0.24
	16 day	0.81 ± 0.16
	19 day	0.99 ± 0.22
	22 day	1.00 ± 0.21
	25 day	1.15 ± 0.23
	28 day	0.90 ± 0.24
Steroid treatment	OVX	1.00 ± 0.19
	E_2	0.50 ± 0.17
	E_2P_4-3h	0.61 ± 0.18
	E_2P_4-6h	1.10 ± 0.06

[a]PreproNPY mRNA levels were measured using 3 μg total RNA isolated from individual rats (6/group) that had been OVX 7–28 days or that had been: a) OVX for the duration of the experiment (9 days; OVX), b) OVX 7 days, then E_2-treated (3 × 150 μg/ml capsules/rat) 2 days (E_2), or c) OVX 7 days, E_2-treated 2 days, then P_4-treated (4 × 50 mg/ml capsules/rat) for 3 or 6 hours (E_2P_4—3 h and E_2P_4—6h, respectively). Data is expressed as mean ± SEM of the ratio of preproNPY mRNA to cyclophilin mRNA (cyclophilin mRNA hybridization is used as an internal control). For the OVX experiment, means then were normalized such that the average of all means equaled 1.0. For the steroid hormone experiment, data were normalized such that the mean of the OVX group equaled 1.0. Data were analyzed by one-way ANOVA and Student-Newman-Keuls' multiple range test.

TABLE 2. In Situ Hybridization for Arcuate Nucleus PreproNPY mRNA[a]

Steroid Treatment	Density	Area	Density* Area
E_2	2.40	2.20 ± 0.2	5.33 ± 0.5
E_2P_4	2.50	2.96 ± 0.4	6.86 ± 0.6

[a]E_2 treatment (3 × 150 μg/ml capsules) was for 2 days beginning 7 days post-OVX and P_4 treatment (4 × 50 mg/ml capsules) was for 6 hours on day 2 of E_2 treatment. ^{35}S-labeled preproNPY cRNA probe was allowed to hybridize with paraformaldehyde fixed, free-floating 30-μm sections through hypothalamus. Sites of hybridization were determined by film and emulsion autoradiography. Film autoradiographs were analyzed by an MCID image analysis system to determine the density of hybridization and area of hybridization of cRNA probe over the arcuate nucleus. Values for the E_2/P_4 animal are different from the E_2 animal for every category at $p < 0.05$ using a one-tailed t test.

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Hypothalamic Neuropeptide Y Expression in Normal and Aberrant Metabolic Homeostasis

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The peripheral and central mechanisms that coordinate and control feeding remain poorly defined. It is now widely believed that neuropeptide Y (NPY) plays an integral role in the normal control of food intake.[1] Thus, if NPY neurons are among the central nervous system (CNS) systems that initiate feeding, then peripheral metabolic status may directly influence the expression and secretion of NPY. We have investigated the regulation of hypothalamic NPY expression, as assessed by measuring preproNPY mRNA levels, in three models of aberrant metabolic homeostasis.

Initially, hypothalamic preproNPY mRNA levels were assessed in animals subjected to food deprivation for up to 72 hours.[2] These studies demonstrated a time-dependent increase in hypothalamic preproNPY mRNA levels in food-deprived animals which reached 2.6-fold above levels found in fed animals (TABLE 1). Using the technique of *in situ* hybridization, we verified that this change in NPY expression was localized to the arcuate nucleus. Next, hypothalamic preproNPY mRNA levels were examined in a model of experimental diabetes mellitus, the streptozotocin-treated rat.[3] Again, hypothalamic preproNPY mRNA levels were increased in diabetic animals compared to controls and this increase could be prevented with insulin replacement (TABLE 1). This increased expression was again localized to the arcuate nucleus. Finally, NPY expression was examined in genetically obese Zucker "fatty" rats.[4] As predicted, preproNPY mRNA content was increased specifically in the hypothalamus of obese male and female rats when compared to their lean littermates (TABLE 1). Interestingly, when the effects of food deprivation on hypothalamic preproNPY mRNA levels were examined in lean and obese animals, only lean animals displayed increased NPY expression (TABLE 1).

Each of these experimental systems share similar changes in peripheral metabolic status, particularly with respect to signals reflecting carbohydrate status. In food-deprived animals, plasma insulin and glucose are depressed while glucocorticoids are elevated. In diabetic animals, plasma insulin is absent, glucose is high, but insulin-dependent glucose utilization is absent and glucocorticoids are elevated. Similarly, obese "fatty" rats are hyperinsulinemic but euglycemic with elevated serum glucocorticoids. When coupled with observations of glucocorticoid regulation of NPY in other systems,[5] these data suggest that glucocorticoids stimulate while insulin inhibits NPY expression, as is seen for the regulation of phosphoenolpyruvate carboxykinase.[6] Similarly, glucose may modulate NPY secretion from hypothalamic neurons in the situation analogous to the regulation of

TABLE 1. Hypothalamic PreproNPY mRNA Content in Aberrant Metabolic Homeostasis[a]

Experimental System	Experimental Group	PreproNPY mRNA
Food deprivation	control	1.0 ± 0.08
	O/N fast	1.4 ± 0.23
	48 hr deprived	2.5 ± 0.41*
	72 hr deprived	2.6 ± 0.68*
Experimental diabetes	control	1.0 ± 0.45
	STZ	4.1 ± 1.5*
	STZ + insulin	1.0 ± 0.18
Zucker fatty rats	lean—male	1.0 ± 0.44
	obese—male	3.2 ± 0.55*
	lean—female	1.0 ± 0.22
	obese—female	2.0 ± 0.19*
	lean—male, fed	1.0 ± 0.23
	lean—male, FD	2.1 ± 0.3*
	obese—male, fed	2.0 ± 0.24
	obese—male, FD	2.6 ± 0.26

[a]Data shown above were taken from REFERENCES 2–4 and other unpublished work. For each experimental system, data are expressed as mean ± SEM relative to the mean value for control animals; for Zucker fatty rats, lean fed animals of the same sex are taken as control. STZ = streptozotocin-treated animals; FD = 72 hr food-deprived animals. For food-deprived normal rats, values for 48 hr and 72 hr food-deprived animals are different from control at $p < 0.05$; for diabetic animals, STZ-treated animals are different from controls at $p < 0.05$; obese males and females are different from lean males and females respectively at $p = 0.015$ and $p = 0.005$; food-deprived lean animals are different from fed lean animals at $p = 0.003$, but food-deprived and fed obese animals show no significant differences. Values reported here for food-deprived and fed obese animals were extrapolated from data in REFERENCE 4 and other unpublished data.

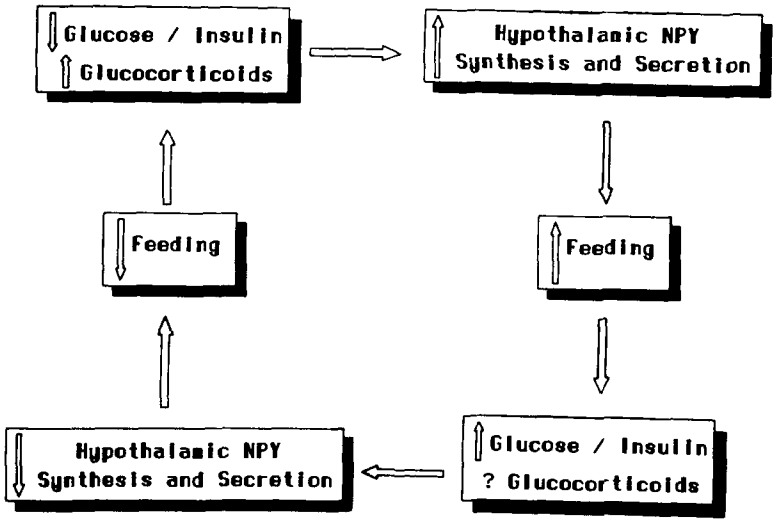

FIGURE 1. Model for regulation of NPY expression by peripheral metabolic status.

somatostatin secretion.[7] Therefore, we can propose a model for the regulation of NPY expression/secretion by peripheral metabolic status (FIG. 1) in which insulin, glucose, and glucocorticoids interact to modulate NPY expression and secretion. This model does not preclude that different signals influence NPY expression vs secretion, nor is it meant to imply that other signals, *e.g.*, other neural systems, are not involved in NPY regulation. The model does provide a framework through which peripheral signals could directly modulate brain function. Experiments are currently in progress to test the separate influences of insulin, glucose, and glucocorticoids on NPY gene expression and secretion in the hypothalamus.

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Central Actions of Neuropeptide Y and Its Related Peptides in the Dog, with Special Reference to Their Effects on the Hypothalamic-Pituitary-Adrenal Axis, Feeding Behavior, and Thermoregulation

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In view of its widespread distribution, very high concentrations, and coexistence with catecholamines in the central nervous system, neuropeptide Y (NPY) has received much attention in the field of neuroscience.[1-3] The role of this peptide in brain function, including neurohormonal or behavioral functions has been studied mainly in laboratory rats. Recent reports have demonstrated that PYY and PP, discovered as gastrointestinal hormones, also occur in the brain, although their physiological roles remain unclear. In this paper, we have summarized the effects of NPY and its related peptides on brain function after intracerebroventricular administration in conscious dogs. We have found that the PP family of peptides have a variety of central actions (TABLE 1), some of which reflect species differences.

Hypothalamic-Pituitary-Adrenal Axis

There is increasing evidence that NPY and its related peptides influence the release of pituitary hormones, including ACTH.[4] Central injections of NPY increase hypothalamic CRF immunoreactivity and plasma ACTH and cortisol concentrations in the rat. In the canine brain, NPY and PYY evoke as strong an ACTH and cortisol secretion as CRF, whereas a C-terminal fragment of NPY, NPY19–36, has much less potency. NPY potentiates CRF-induced ACTH release, and the ACTH response to NPY is attenuated by a CRF antagonist. Pretreatment with anti-NPY γ-globulin reduces the ACTH and cortisol responses after hypoglycemic stress. These results, together with strong densities of NPY and its receptors in the hypothalamus and with high concentrations of NPY in the pituitary

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portal blood, indicate that NPY may modulate ACTH secretion in concert with CRF. The ACTH-releasing activity of NPY is also shared by PP. Since NPY and PYY receptors discern this peptide, PP possibly exerts the effect via its putative receptors in the brain. In the dog, stimulation of a central catecholaminergic system has been reported to inhibit ACTH secretion.

Feeding Behavior

NPY is the most potent naturally occurring orexigenic agent yet described in various species, including rats, mice, ground squirrels, pigs, sheep, and chickens. PYY is also effective in stimulating food intake. However, neither centrally administered NPY, PYY, nor norepinephrine initiates feeding in satiated dogs, suggesting species differences in central appetite regulation.[5] In contrast to the absence of an orexigenic effect of NPY and PYY, porcine (canine) PP stimulates feeding in a dose-dependent manner in this species. Human PP also acts in the same way, but its C-terminal fragments, PP18–36 and PP23–36, do not, suggesting that the whole PP molecule is required for its full effect on appetite stimulation. PP also shows weak, but significant orexigenic activity when administered into the rat brain.

TABLE 1. Central Actions of PP Family Peptides in the Dog

Central Actions	NPY	PYY	PP	Order of Magnitude
1. Effect on hypothalamic-pituitary-adrenal axis				
Stimulation of ACTH and cortisol secretion	+[a]	+	+	PYY ≥ NPY ≥ PP
2. Effect on appetite regulation				
Stimulation of feeding	−	−	+[b]	PP >> NPY = PYY
3. Effect on thermoregulation				
Decrease of rectal temperature	+	+	−	PYY ≥ NPY >> PP
Attenuation of PGE_2-induced hyperthermia	+		−	NPY >> PP

[a]NPY19–36: ±.
[b]PP18–36: ±.

Thermoregulation

NPY induces hypothermia in a normal state and PYY has an even more potent and prolonged effect.[6] In addition to this, NPY attenuates the fever response produced by central administration of prostaglandin E_2. However, PP does not alter the rectal temperature in either situation. Therefore, the hypothermic effects of NPY and PYY are likely to be mediated by their receptors, and not to be primarily concerned with ACTH secretion, because there is no effect of PP on thermoregulation. There is evidence that catecholamines act to accelerate heat loss or block heat production in the preoptic anterior hypothalamus. It is thus possible that NPY works, at least in part, with catecholamines in the brain. In rats, NPY was reported to have no or minimal effect on body temperature.

To summarize, not only NPY but its structurally related peptides, PYY and PP, have multifunctions in the canine brain, including activation of the hypothalamic-pituitary-adrenal axis, stimulation of feeding behavior, and production of hypothermia. The PP family of peptides could act via their own receptors, both in concert with, and independent from, catecholamines. It is important that the species differences are taken into account when studying the neuroendocrine or behavioral effects of NPY and its related peptides.

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Effects of Neuropeptide Y and Its Related Peptides on Feeding and Learning Behaviors in the Mouse

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It was previously reported that neuropeptide Y (NPY), a major neuropeptide in the brain, increases feeding in many species and improves learning in mice.[1,2] Its related peptides, PYY[3] and PP,[4] have also been shown to exist in the brain and have orexigenic effects.[5–7] PYY and PP are indeed the most potent orexigenic substances in rats[5] and dogs,[7] respectively. However, it is not known whether PYY and PP have any effects on learning behavior. In this study, we compared the effects of NPY, PYY and PP on the feeding and learning behaviors of the mouse in order to study structure-activity relationships.

A simple new technique which allows easy injection into the cerebroventricle at any desired moment and frequency, was developed and used. Male mice of the ddY strain were implanted with cannula at 9–10 weeks of age, and subjected to the following learning and feeding tests 1 week later.

Learning Study

Learning behavior was estimated in step-down-type passive avoidance in which an apparatus delivered a scramble-type electric shock of 0.25 mA for 1 sec 3 times each 5 sec. Mice were intracerebroventricularly (icv) given NPY, PYY and PP (0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10 μg/brain) dissolved in 4 μl of artificial cerebrospinal fluid (ACSF) immediately after training. Step-down-latency (SDL) was tested twice at a 30–60-sec interval 24 hr later, and the longer SDL was recorded. As shown in FIGURE 1, NPY, PYY and PP significantly improved memory retention at a dose of 0.03, 0.3 and 3.0 μg, respectively, when compared with the ACSF group.

Feeding Study

Food intake was examined during 4 successive periods of 0–20 min, 20–60 min, 1–2 hr and 2–4 hr after icv treatment which began between 10 a.m. and noon. NPY, PYY and

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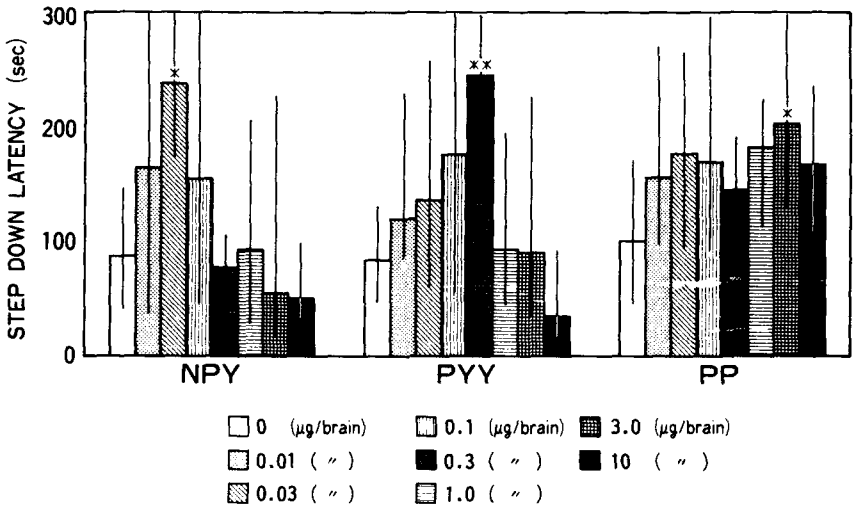

FIGURE 1. The effects of NPY, PYY and PP on memory consolidation in the mouse. Each column indicates the median step-down-latency (SDL) of 14–15 mice. Significance from 0 μg/brain dose was determined by Mann-Whitney's U-test. *: $p < 0.05$; **: $p < 0.01$.

PP significantly increased food intake 60 min after administration at doses of 3.0 (0.3–10), 1.0 (0.1–3.0) and 1.0–10 μg, respectively (FIG. 2). Mice which received higher doses of NPY and PYY appeared sedated during the first session (0–20 min).

NPY shares a high sequence homology with PP (50%) and even more so with PYY

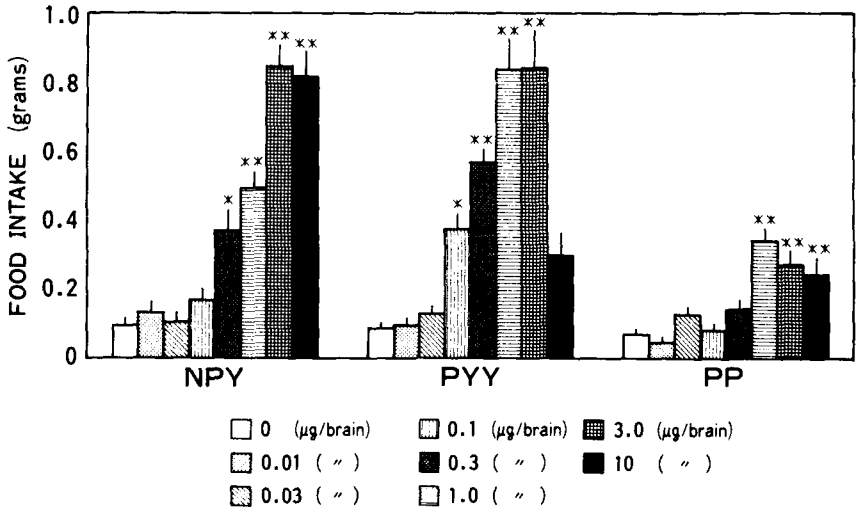

FIGURE 2. The effects of NPY, PYY and PP on food intake in the mouse (0–1 hr). Each column indicates the mean food intake of 15 mice. Significance from 0 μg/brain was determined by Dunnett's test. *: $p < 0.05$; **: $p < 0.01$.

(70%). PYY generally mimics the actions of NPY, but to a greater extent. In addition to this, the similarities in the pharmacological and structural characteristics of NPY and PYY receptors suggested that they regulate brain function through interaction at common receptor sites.[8] Utilizing a bioassay, two distinct subtypes of NPY receptor are postulated;[9] the Y1-type (postsynaptic) the effects of which could be seen by the complete NPY molecule, and the Y2-type (presynaptic) the effects of which could be identified by even long C-terminal fragments. The difference in the effects of NPY and its C-terminal fragments between feeding and learning behaviors suggested that these behaviors are produced through the Y1 and Y2 receptors, respectively.[10] In the present study, we found that among the PP family peptides, the ranking of potency in improving memory retention was NPY > PYY > PP, whereas PYY > NPY > PP in stimulating feeding in mice. These results suggest that the NPY/PYY receptors which mediate learning and feeding behaviors are different. It may thus be possible to selectively enhance memory processes or increase food intake through the NPY (Y2) or PYY receptors, respectively. The heterogeneity of brain PYY receptors needs to be clarified.

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Evidence That Neuropeptide Y Elicits Eating by Acting in the Caudolateral Paraventricular/Perifornical Hypothalamus[a]

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Previous work has shown that neuropeptide Y (NPY) is a powerful stimulant of eating behavior,[1] with acute central injections producing a dramatic eating response and chronic injections producing hyperphagia and obesity.[2,3] These effects appear to be due, at least in part, to NPY's actions within the hypothalamus, since injections of NPY directly into this structure are highly effective, whereas injections bracketing this structure are ineffective.[4] To date, however, there is no clear indication that any particular hypothalamic structure is most sensitive to the eating-stimulatory effects of NPY, with injections into relatively widespread hypothalamic areas producing essentially equivalent responses.[4] The goal of the present study was to identify the hypothalamic site(s) most sensitive to NPY.

To accomplish this, NPY (78 pmole) was injected through indwelling guide cannulas into an array of 47 different hypothalamic sites (n = 2 to 11 rats/brain site) and the food intake of satiated adult male rats was measured 1 and 4 hours postinjection. To minimize the diffusion of NPY away from the injection site, it was injected in an extremely small volume (10 nl) of artificial CSF. Each subject received a minimum of five tests, 4 with NPY and 1 with vehicle. An additional group of 20 rats was used to investigate the extent of NPY diffusion away from the injection site. To accomplish this, these subjects were given hypothalamic injections of ^{125}I-NPY dissolved in 10 nl of artificial CSF. Subsequently they were sacrificed, their brains were frozen and cut in 100-μm-thick coronal sections, and the radioactivity of each section was determined in a gamma counter.

The behavioral results demonstrate that NPY's stimulatory effect on eating behavior is site specific, with by far the largest responses obtained in a small brain area centered on the medial perifornical hypothalamus at the level of and encompassing the caudolateral tip of the paraventricular nucleus. The average increase in food intake of the 11 animals with injections within this region was 12.5 ± 1.5 grams in 1 hour, and 20.0 ± 2.7 grams in 4 hours. Injections of NPY into sites bracketing this region, less than 1 mm from the critical area, yielded reduced responsiveness, from 25% to 84% lower in magnitude, with even smaller responses at greater distances. Analysis of the distribution of the injected

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^{125}I-NPY revealed that the diffusion was extremely restricted, with 100% of the NPY localized within 1.0 mm of the injection and 95% found within 0.5 mm of the injection site.

These findings suggest that the receptors activated by NPY to induce eating are centered in the medial perifornical region of the hypothalamus at the level of and including the caudolateral tip of the paraventricular nucleus. It may be noteworthy that even though this region encompasses the caudolateral tip of the paraventricular nucleus, it is clearly distinct from the more anteromedial portion of the paraventricular nucleus, the site where norepinephrine, another feeding stimulatory neurotransmitter, is most effective.[5]

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Chronic and Continuous ICV Infusion of Neuropeptide Y Disrupts the Nycthemeral Feeding Patterns in Rats

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INTRODUCTION

Neuropeptide Y (NPY) has a potent stimulatory effect on food intake[1,2] and particularly on carbohydrate intake.[3] We previously showed that the obese hyperphagic Zucker rat is characterized by significant increases in NPY content in several brain nuclei and particularly in hypothalamic nuclei involved in the regulation of food intake.[4] This was observed in the paraventricular nuclei, one of the main sites of action of NPY[5] as well as in the suprachiasmatic nuclei which is actually considered to be the principal circadian oscillator of the rodent brain[6] and is largely involved in the regulation of the feeding periodicity.[7] We therefore hypothesized that hyperphagia and disturbed nycthemeral meal pattern of the obese Zucker rat might be related to the high hypothalamic NPY levels. To test this hypothesis, we tried to reproduce this physiological situation and to simulate these high central levels of NPY by continuously infusing NPY in the brain ventricle of Long-Evans rats through osmotic minipumps.

MATERIALS AND METHODS

Male Long-Evans rats (Centre d'Elevage R. Janvier. Le Genest-Saint Isle, France) weighing 336.7 ± 6.3 g were housed in individual wire cages in an air-conditioned room with an automatic light-dark cycle (dark between 9.30 and 20.00). The animals were fed on a high fat (HF) diet (68% of energy from fat) and a high carbohydrate (HC) diet (69% of energy from carbohydrates) which were given simultaneously in two separate cups. Diets and tap water were available ad libitum. After 7 days of habituation to these conditions, the rats were implanted with a stainless steel guide cannula (27 gauge; Terumo Corporation, Leuven, Belgium) aimed in the right lateral ventricle. After 7 days of recovery, the rats were implanted with an osmotic minipump (Alzet Model 2002; Palo Alto, USA) under light ether anesthesia. The pumps were placed subcutaneously on the rat's back between the shoulders. Prior to implantation, the pumps were filled with either sterile artificial cerebrospinal fluid (CSF; passed through 0.22 micron Millipore filters; n = 11) or NPY dissolved in CSF (n = 11). They were incubated at 37°C during 4 hours to avoid a premature excessive release induced by the difference in temperature existing between surgery room and rat body. NPY concentration in the pump was 1 μg/μl and the pump delivered 0.44 μg/hr during 14 days. The pumps were removed after 2 weeks under light ether anesthesia. Rats were killed by decapitation one week later.

Food intake was recorded twice daily at the end of light and dark periods from the first day with food choice (7 days before cannulation) until sacrifice. Body weight was recorded daily during the same period.

RESULTS AND DISCUSSION

Body weight and food intake are shown in FIGURE 1. Rats infused with NPY were significantly heavier than those infused with CSF. This was partly due to the increase of the fat mass since at autopsy, it appears that abdominal fat mass was very developed in the NPY rats. These results confirm a previous experiment using repeated acute NPY injections.[8] Total food intake was also significantly increased by NPY infusion (21.0 ± 1.6 vs 11.5 ± 0.5 g/day; $p < 0.01$). The effect of NPY infusion was more marked on the HC intake (+191%) than on the HF intake (+63%). This increase in quantity was associated with a modified distribution of food intake between the light (L) and dark (D) periods (FIG. 2). The D/L ratio of HC intake was 4.3 ± 0.4 during the preinfusion period and fell to 1.6 ± 0.1 ($p < 0.001$) during NPY infusion. This effect lasted during the entire infusion period with ratios reaching nearly 1 during the first days. A parallel fall of the D/L ratios of HF intake was observed during the two first days of infusion. The end of infusion was immediately followed by an increase of both ratios.

These results not only showed that NPY has a stimulatory effect on food intake with a preferential effect on carbohydrate intake but also that it can modify the feeding pattern during all the time where its central levels are high. They agree with our hypothesis of a very important role of NPY in the development of hyperphagia and obesity in some animal models like the Zucker rat.

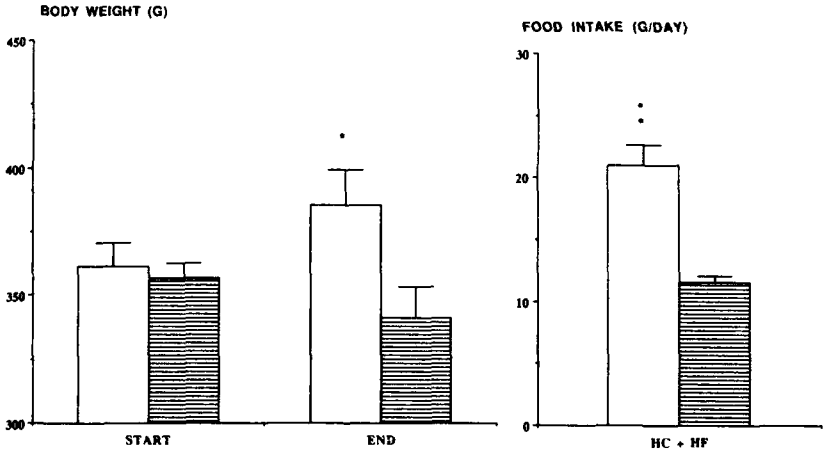

FIGURE 1. Body weight at the start and end of infusion and total food intake (high carbohydrate (HC) diet + high fat (HF) diet) of rats infused with either neuropeptide Y (*open bars;* n = 11) or vehicle (cerebrospinal fluid; *stripped bars;* n = 11) during 14 days through an osmotic minipump. *: $p < 0.05$; **: $p < 0.01$.

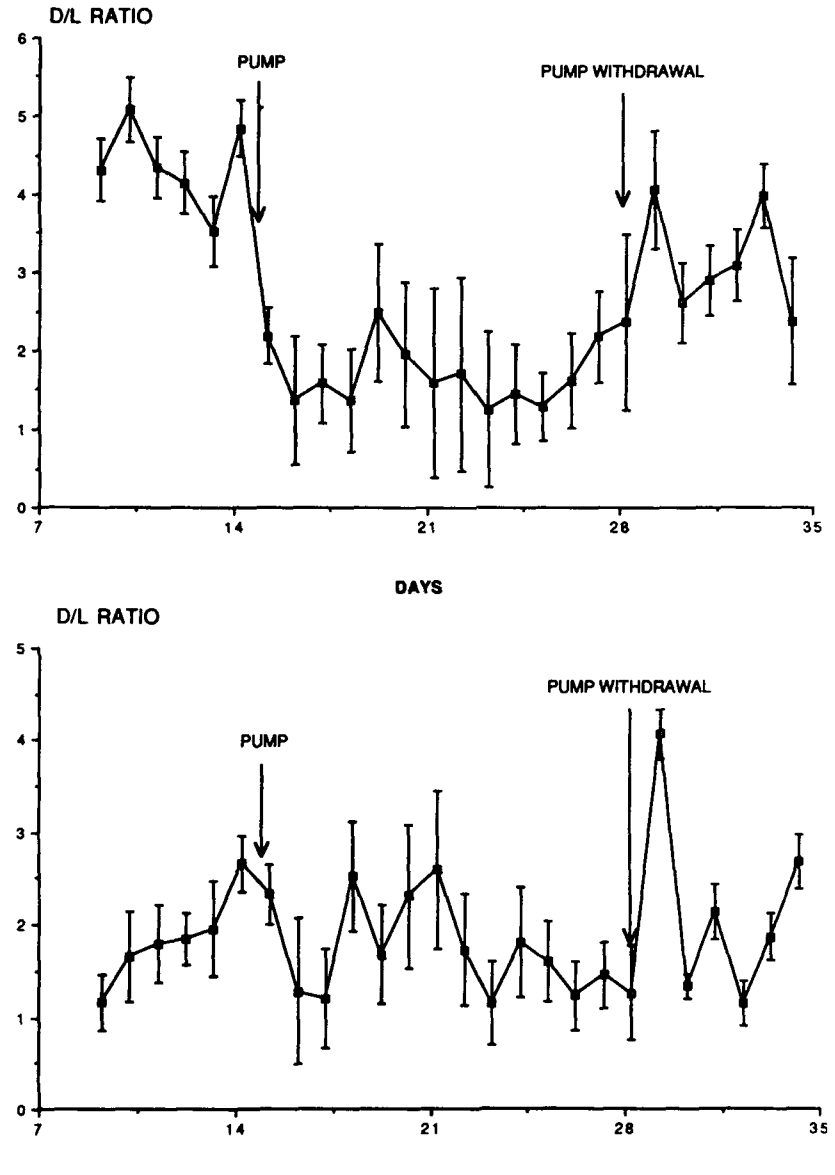

FIGURE 2. Distribution between the dark (D) and light (L) periods of diet intake for the high carbohydrate (HC) diet *(top panel)* and high fat (HF) diet *(bottom)* in rats infused with neuropeptide Y (0.44 μg/hr during 2 weeks).

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Neuropeptide Y in Pediatric Neural Crest Tumors

Correlation with Malignancy, Metastases, and Clinical Outcome

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Neuropeptide Y (NPY) is a 36-amino acid regulatory peptide in the human peripheral sympathetic and central nervous systems. Elevated concentrations of NPY-like immunoreactivity (NPY-LI) have been found in plasma and tumor tissue of patients with tumors of the neural crest.[1] We have previously reported high plasma concentrations of NPY-LI in children with neuroblastoma.[2,3]

Neuroblastoma (NBL) is a malignant pediatric tumor of the sympathetic nervous system with poor prognosis if metastatic disease is found over one year of age. Infants with NBL and those with localized disease have better prognosis. Children with benign neural crest tumors, ganglioneuroma (GN), and potentially malignant, ganglioneuroblastoma (GNB), most often do well.

We have studied the relation of NPY to tumor malignancy, metastatic disease, and clinical outcome in 17 pediatric patients with neural crest tumors, 10 children with NBL, 4 GNB, and 3 GN. NPY-LI was analyzed in extracted plasma and tumor tissue using a competitive radioimmunoassay.[4]

At diagnosis plasma NPY-LI was increased ($p < 0.001$) in NBL children (270; 110–640 pmol/L, median; interquartile range) when compared to 17 matched healthy controls (34; 27–52 pmol/L) and 34 pediatric tumor controls (27;20–37 pmol/L) (FIG. 1). The highest concentrations were found in four children with metastatic disease (Evans stage IV) (360,640,760, and 850 pmol/L resp).

Children with GN and GNB had lower levels (66; 44–70 pmol/L) but still higher than the controls ($p < 0.05$ and $p < 0.001$ resp) (FIG. 1). In all children plasma NPY-LI normalized during treatment. Seven children relapsed, all had increasing plasma NPY-LI. In three children the increase in plasma NPY-LI preceeded all other signs of relapse. The five children who died had higher ($p < 0.01$) concentrations of NPY-LI at diagnosis than the survivors (FIG. 1).

Higher concentrations of NPY-LI were found in NBL tissue (220 and 144 pmol/g) than in GN tissue (0.6 pmol/g) and healthy adrenal (5.5 pmol/g) (FIG. 2). In NBL metastasis NPY-LI was considerably increased compared to primary tumor in the same child (3091 vs 220 pmol/g) (FIG. 2).

During surgical tumor manipulation plasma NPY-LI increased 2–4 times in NBL patients while during surgery of GN plasma NPY-LI remained normal.

Gel-permeation chromatography revealed a majority of NPY-LI corresponding to intact NPY in NBL primary tumor while metastasis and plasma in the same patient mainly contained smaller immunoreactive fragments.

We conclude that plasma NPY-LI is increased at diagnosis in children with neural crest tumors. The highest levels are found in those with malignant tumors and advanced

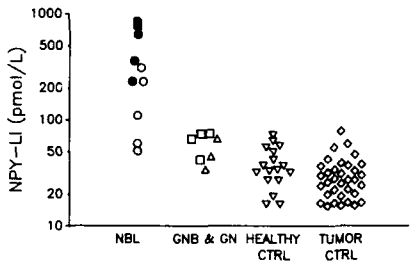

FIGURE 1. NPY-LI in plasma at diagnosis in children with neural crest tumors and matched healthy controls and children with other malignant tumors. NBL = neuroblastoma, GNB = ganglioneuroblastoma *(squares)*, GN = ganglioneuroma *(triangles)*. *Solid symbol* = patient deceased.

metastasizing disease. Plasma NPY-LI may be an important marker for diagnostic and staging purposes. High plasma NPY-LI at diagnosis indicate bad prognosis. Furthermore, plasma NPY-LI may be a sensitive marker for monitoring disease and detecting early relapse.

Tumor concentrations of NPY-LI are higher in malignant tumors with a further increase in metastatic tissue indicating a close relation of NPY with aggressive tumor behaviour. The presence of smaller fragments of NPY in plasma and metastasis implicate a disturbed production or metabolism of NPY in rapidly growing tumor cells. Since small C-terminal fragments of NPY may modulate the vasoconstrictive effect of intact NPY[5] these fragments could be an explanation of the absence of hypertension, even during surgery, in most children with NBL.

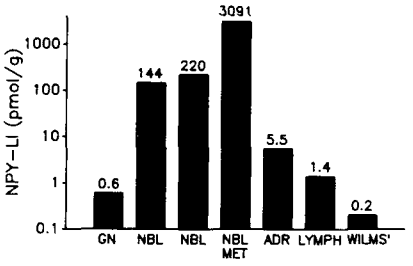

FIGURE 2. NPY-LI in tumor tissues and control tissues in pmol/g wet weight. NBL = neuroblastoma, GN = ganglioneuroma, MET = metastasis, ADR = healthy adrenal, LYMPH = lymphoma, WILMS' = Wilms' tumor.

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Reduction of Neuropeptide Y-Induced Feeding in Tumor-Bearing Rats[a]

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The potency of exogenously-administered neuropeptide Y (NPY) in stimulating feeding suggests an involvement of endogenous NPY in the normal control of food intake. Therefore, alterations in the feeding response to NPY or in endogenous levels of NPY might be associated with various feeding pathologies. Specifically, we hypothesized that anorectic tumor-bearing (TB) rats would exhibit reduced feeding following the intrahypothalamic injection of NPY. To test this hypothesis, 24 ga stainless steel cannulae were implanted into the paraventricular hypothalamic area of 27 anesthetized (pentobarbital 45 mg/kg) male Fischer 344 rats (250–300 g) as reported previously.[1] Two weeks later 15 of these rats were inoculated (sc) with 50 mg of fresh methylcholanthrene-induced sarcoma, while the remaining rats received sham inoculations. Since this tumor line typically causes significant anorexia during the third week of tumor growth,[2] tests of NPY-induced feeding were scheduled prior to (days 14 and 18), at the onset of (days 21 and 25) and during (days 28 and 35) tumor-induced anorexia. One group of TB ($n = 8$) and control ($n = 7$) rats received 2 μg of NPY contained in 1 μl of artificial cerebrospinal fluid (CSF) at these times. The remaining TB ($n = 7$) and control ($n = 5$) rats received 1 μl injections of CSF only. Rat chow and water intake were assessed for 4 hr following these injections. Body weight was monitored daily as was the ad libitum intake of rat chow and water. At the conclusion of the experiment the rats were decapitated and the tumors were weighed (83 ± 4 g). Plasma was retained for the determination of NPY concentrations by radioimmunoassay.[3] Statistical significance was determined by analysis of variance techniques.

As illustrated in FIGURE 1, consistent significant ($P < 0.01$) anorexia was first observed 25 days after tumor inoculation. Although there was no difference in 4-hr feeding response between TB and control rats following the injection of NPY on day 14 (FIG. 2), tests conducted on day 18 and subsequently revealed significant ($p < 0.05$) reductions in NPY-induced feeding in TB rats. NPY stimulated feeding was also reduced in TB rats by 39% and 48% during the 1 and 2 hr measurements, respectively. Thus, even prior to the onset of anorexia, the feeding response to NPY was reduced in TB rats. NPY-induced feeding became even more attenuated in TB rats as the anorexia worsened, with day 34 values being reduced by 73%. Plasma concentration of NPY was reduced significantly ($p < 0.05$) by 30% in TB rats (12.3 ± 1.2 vs 17.7 ± 1.1 ng/ml), suggesting either reduced synthesis or increased degradation of this peptide peripherally in these rats.

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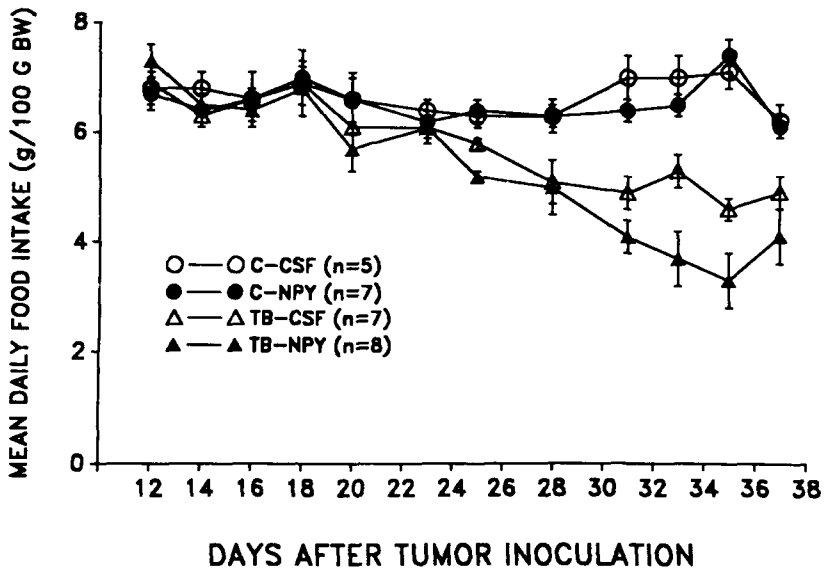

FIGURE 1. Mean (± SEM) daily ad libitum intake of rat chow by tumor-bearing (TB) and control (C) rats treated with intrahypothalamic injection of artificial cerebrospinal fluid (CSF) or neuropeptide Y (NPY).

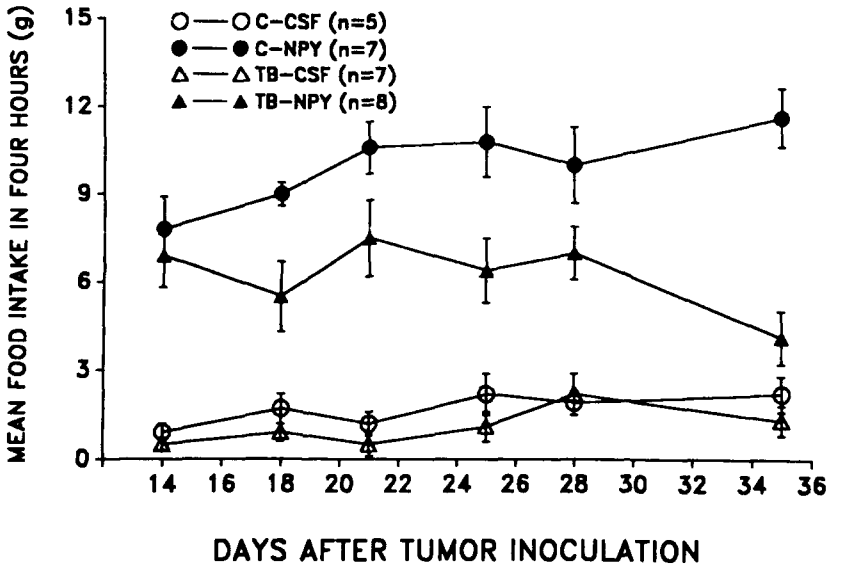

FIGURE 2. Mean (± SEM) intake of rat chow by tumor-brearing (TB) and control (C) rats during the four hours following the intrahypothalamic injection of artificial cerebrospinal fluid (CSF) or 2 μg of neuropeptide Y (NPY).

These results indicate that the mechanisms of NPY-induced feeding are altered by the presence of a tumor. The reduction of NPY-induced feeding prior to the onset of anorexia suggests that the alteration of these feeding mechanisms may be involved in the etiology of cancer anorexia. The decrease in plasma NPY concentration in TB rats supports this hypothesis, suggesting that cancer may deplete NPY concentration to less than that necessary to control feeding normally. Alternatively, other factors[2] that may cause cancer anorexia may also reduce the feeding response to NPY as well as endogenous NPY levels in TB organisms.

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Changes in Hypothalamic Neuropeptide Y during Immunization in the Rat

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INTRODUCTION

Several studies suggest that both the sympathetic nervous system and the brain are involved in the immune response: (i) signals from the activated lymphoid cells produce an important increase in electrical activity in the ventromedial nucleus of the rat hypothalamus (HY),[1] and (ii) the decrease of norepinephrine in the rat spleen during immune response is considered as a sympathetic signal regulating this response.[2] Moreover, changes of some circulating hormones, such as an increase in the plasma levels of corticosterone, are observed after antigenic stimulation of rats.[2] A hypothetical schema of the neuro-hormonal regulation during activation of the immune system is proposed in FIGURE 1. Neuropeptide Y (NPY) and catecholamines (CA) were measured in the central nervous system (HY and cortex) and in the plasma of the rat to study their involvement in this schema.

MATERIALS AND METHODS

Twenty-eight rats were injected i.p. with 5.10^9 human red blood cells in 1 ml 0.9% NaCl, as antigen; 14 rats were injected i.p. with 1 ml 0.9% NaCl (controls). Four immunized rats and 2 controls were sacrified from the 2nd day to the 14th day after immunization at two-day intervals. CA (norepinephrine = NE, epinephrine = E, dopamine = DA) were differentially measured by a HPLC.EC method after purification using alumina. NPY was measured by an IRMA using two monoclonal antibodies.[3] Student's *t* test was used for the statistical analysis

RESULTS (TABLE 1)

In the plasma, (i) up to the 6th day after immunization, a slight decrease was observed in all the parameters without statistical significance, (ii) NPY was significantly increased from the 8th day ($p < 0.01$) with a maximal level on the 10th day; the concentration returned to the basal range on the 12th day, and (iii) plasma NE was significantly increased on the 8th day ($p < 0.05$) and returned to the normal on the 10th day. No significant variation of other CA was observed.

In the cerebral tissues, (i) HY content of NPY was significantly increased from the 6th day ($p < 0.05$) and reached a maximum on the 10th day ($p < 0.01$); the basal level was

recovered on the 12th day, (ii) within the first 8 days after immunization, HY NE, E and DA contents were decreased by 20%; an increase in the 3 CA was observed on the 10th day simultaneously with the increase in NPY, with a normalization on the 12th day, and (iii) no variation was found in NPY and CA contents on the cerebral cortex.

DISCUSSION

These results allow to conclude that (i) the NPY content of HY is increased from the 6th day to the 12th day after immunization. In comparison, the increase in CA content of this tissue starts with a delay of 4 days relatively to NPY, but both responses are normalized at the same time. (ii) In plasma, the increase in NPY occurs two days after that observed in HY, but both values return to basal concentration at the same time. As in HY,

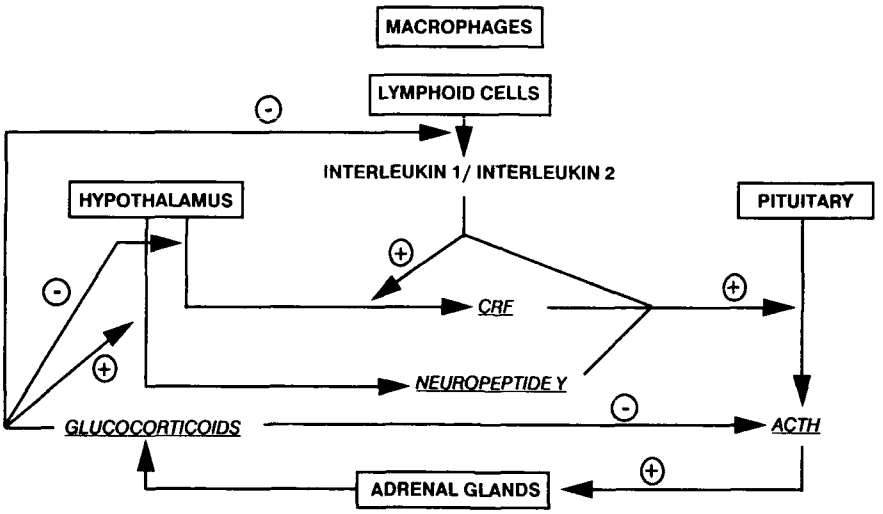

FIGURE 1. Hypothetical place of neuropeptide Y in the modulation of the immune response.

NE levels are more briefly increased. It is noteworthy that the maximal increases of NPY in blood and HY are simultaneously observed at day 10. That indicates a possible ubiquitary involvement of sympathetic and central NPY in the course of immunization. The simultaneous changes in NE suggest that the NPY variations are due to its neuromodulating function; that does not exclude the possible role of NPY as a specific agent in the regulatory processes of immunization. Indeed, the presence of glucocorticoid receptors in HY and the increased corticosterone plasma levels observed in the rat during immunization are well known, so that it can be thought that the NPY syntheses can be stimulated by the glucocortoids produced by immunization according to the sequential way comprizing interleukins, CRF and ACTH. Further studies remain to be done in order to determine if plasma NPY can be considered as a marker of immune response and to delineate its role precisely in immunoregulation.

TABLE 1. NPY Concentrations in Hypothalamus and Plasma during 14 Days after Immunization of the Rat[a]

	Days after Immunization							
	0	2	4	6	8	10	12	14
Hypothalamic NPY	14.9 ± 1.1	16.1 ± 1.1	15.5 ± 2.2	19.3 ± 1.6	20.8 ± 1.6	22.3 ± 1.1	14.4 ± 1.5	15.9 ± 2.0
Plasma NPY	330 ± 83	315 ± 113	ND	303 ± 128	707 ± 288	820 ± 329	390 ± 145	320 ± 121

[a]Expressed as pmoles/g tissue ± s.d. or pmoles/L ± s.d.

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Localization, Characterization, and Neuroendocrine Action of Neuropeptide Y in the Trout Brain

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Neuropeptide Y (NPY) was first isolated from the porcine brain.[1] Immunocytochemical studies have demonstrated that NPY is widely distributed in the brain of mammals[2-4] and nonmammalian vertebrates.[5] However, very few studies have been conducted to investigate the occurrence and the neuroendocrine functions of NPY in fish. This report describes the distribution and biochemical characterization of NPY in the trout brain, as well as the action of NPY on the secretion of gonadotropins *in vivo* and *in vitro*.

Mapping and Characterization of NPY in the Trout Brain

Using a specific antiserum against synthetic porcine NPY,[3] we examined the distribution of NPY-like immunoreactivity in the brain and pituitary of the trout *Salmo gairdneri* with indirect immunofluorescence and peroxidase-antiperoxidase techniques. The highest density of NPY-immunoreactive elements was found in the basal telencephalon and in the hypothalamus. In particular, NPY-immunoreactive neurons were located in the nucleus entopeduncularis (FIG. 1a) and in the preoptic nucleus (FIG. 1b). NPY-immunoreactive fibers were observed throughout the trout brain. The preoptic nucleus, the suprachiasmatic nucleus, and the nucleus entopeduncularis (FIG. 1a) were densely innervated. In addition, NPY-positive fibers were detected in the nucleus lateralis tuberis and the distal and intermediate lobes of the pituitary. The distribution of NPY-containing neurons and fibers in the brain and pituitary of the trout is in good agreement with that recently reported in the goldfish[6] and the dogfish.[7] The NPY-like peptide present in the trout brain was characterized by combining high performance liquid chromatography (HPLC) analysis and radioimmunological detection. Serial dilutions of trout hypothalamus and pituitary extracts produced displacement curves which were parallel to the standard curve. HPLC analysis resolved a major peak which was slightly less hydrophobic

than porcine NPY. These results are consistent with those obtained in frog,[5] goldfish,[6] and sharks,[7] indicating that, in cold-blooded vertebrates, NPY exhibits a shorter retention time than porcine NPY.

Neuroendocrine Action of NPY

An *in vitro* perifusion technique was used to investigate the effect of NPY on gonadotropin (GTH) release from the trout pituitary. When vitellogenic females were used as tissue donors, exposure of the pituitary gland to synthetic porcine NPY (10^{-7} M) caused a slight but significant inhibition of GTH secretion.[8] It has also been reported that, in frogs and toads, NPY causes inhibition of α-MSH release from pituitary melanotrophs.[9,10] In contrast, when NPY was administered to pituitary glands from ovulated

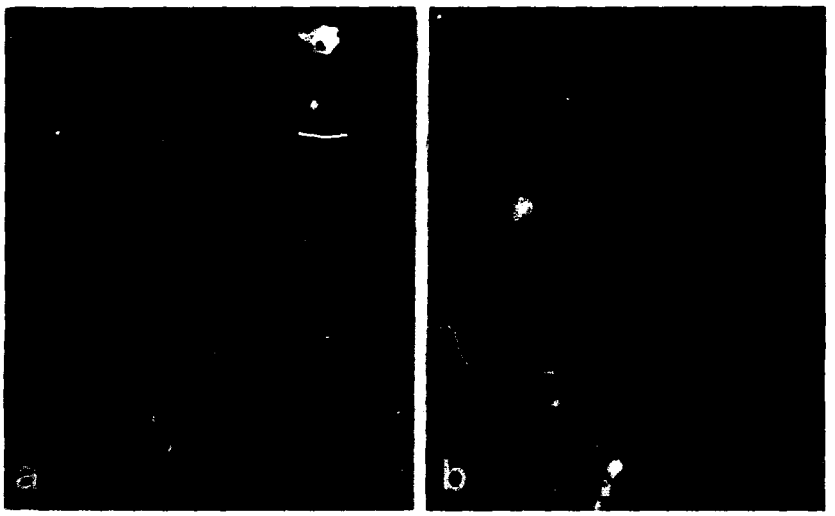

FIGURE 1. Localization of NPY-immunoreactive neurons in the trout brain. **(a)** Perikarya and fibers in the nucleus entopeduncularis. Transverse section. × 320. **(b)** Sagittal section through the anterior part of the preoptic nucleus showing typical NPY-immunofluorescent perikarya. × 420.

females, a robust stimulation of GTH secretion was observed. The greatest stimulation occurred with pituitaries taken on the day of ovulation; in these conditions, the stimulatory effect of NPY was similar to that observed with gonadotropin-releasing hormone (GnRH) (FIG. 2). Vitellogenic females were treated with 1-4-6 androstadien 3-7 dione (PD), an inhibitor of aromatase activity; pituitaries taken from these animals exhibited the same type of response to NPY as ovulated females, *i.e.*, stimulation of GTH release. When pituitaries from ovulated females were exposed to prolonged infusion of the GnRH antagonist D-Phe$^{2-6}$-Pro^{3}-GnRH (10^{-6} M; 30 min), the effect of NPY (10^{-7} M; 15 min) was totally abolished.

Altogether, these data suggest that NPY, contained in nerve fibers terminating directly in the pituitary gland, causes presynaptic modulation of GnRH release in fish. The effect of NPY (inhibitory in vitellogenic and stimulatory in ovulated animals) appears to depend on the *in vivo* steroidogenic environment of the animals.

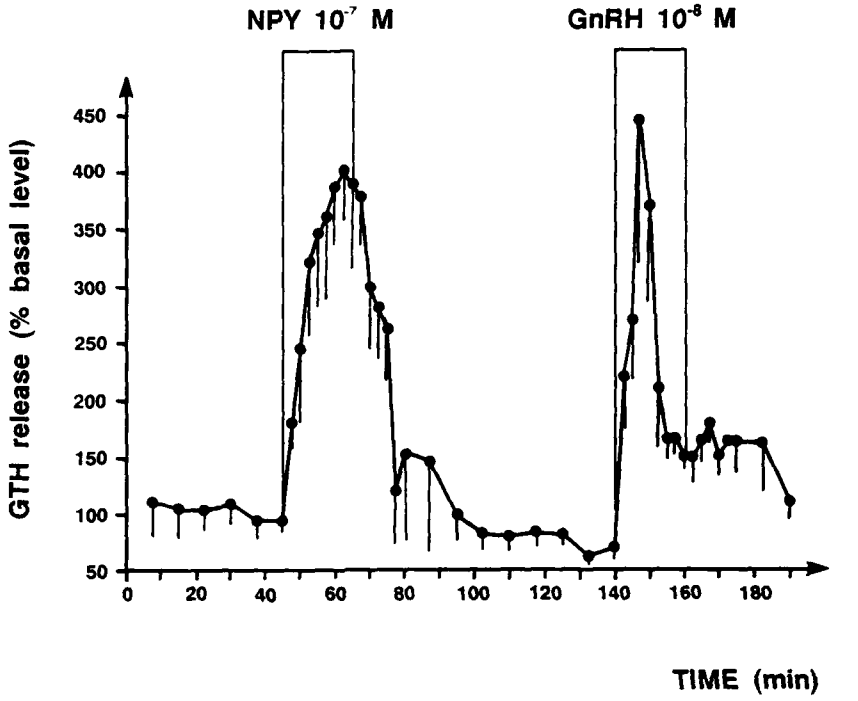

FIGURE 2. Effect of GnRH (10^{-7} M) and NPY (10^{-7} M) on GTH release from perifused trout pituitaries. The pituitary pars distalis were taken within 24 h following ovulation. Synthetic GnRH and NPY were administered for 20 min (*bracketed area*). The profile represents the mean ± SEM of 4 independent experiments.

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Anatomical Distribution and Biochemical Characterization of Neuropeptide Y-Like Immunoreactivity in the Cat Brain and Pituitary[a]

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The various physiological effects induced by the administration of neuropeptide Y (NPY) in the central nervous system (for review see REFS. 1, 2) find an anatomical basis in the wide distribution of this peptide in the brain and spinal cord. In mammals[3-7] the tele- and diencephalon are richly supplied with NPY-immunoreactive (NPY-IR) fibers and cell bodies, whereas the brainstem shows a more contrasted distribution of the immunoreactivity. These data are confirmed by RIA measurements of the NPY concentrations in different brain regions.[8,9]

In this work we studied the anatomical distribution of NPY-immunoreactivity in the cat brain and hypophysis. In addition, we characterized the immunoreactive (IR) products by HPLC analysis and measured the NPY concentrations in a few selected areas. Young animals (5 weeks to 6 months) were chosen because of their high IR content. Five weeks is a time when the majority of the NPY systems should have reached an adult pattern, if we refer to the study by Wahle and Meyer[6] on the visual cortex. Detailed descriptions of the distribution of the NPY neurons in specific areas of the adult cat brain can be found in recent studies by other workers.[10-14]

The immunofluorescence reactions were performed on frontal frozen sections from brains perfused with paraformaldehyde-lysine-periodate fixative, in the absence or after a colchicine injection in the lateral or third ventricle. The anti-NPY antiserum No. 4603-2[3] was used at a concentration of 1:800 over 1 h. This antiserum was shown not to recognize BPP, PYY and FMRF-amide.[15,16]

The tele- and diencephalon were by far the most abundantly supplied with NPY-IR

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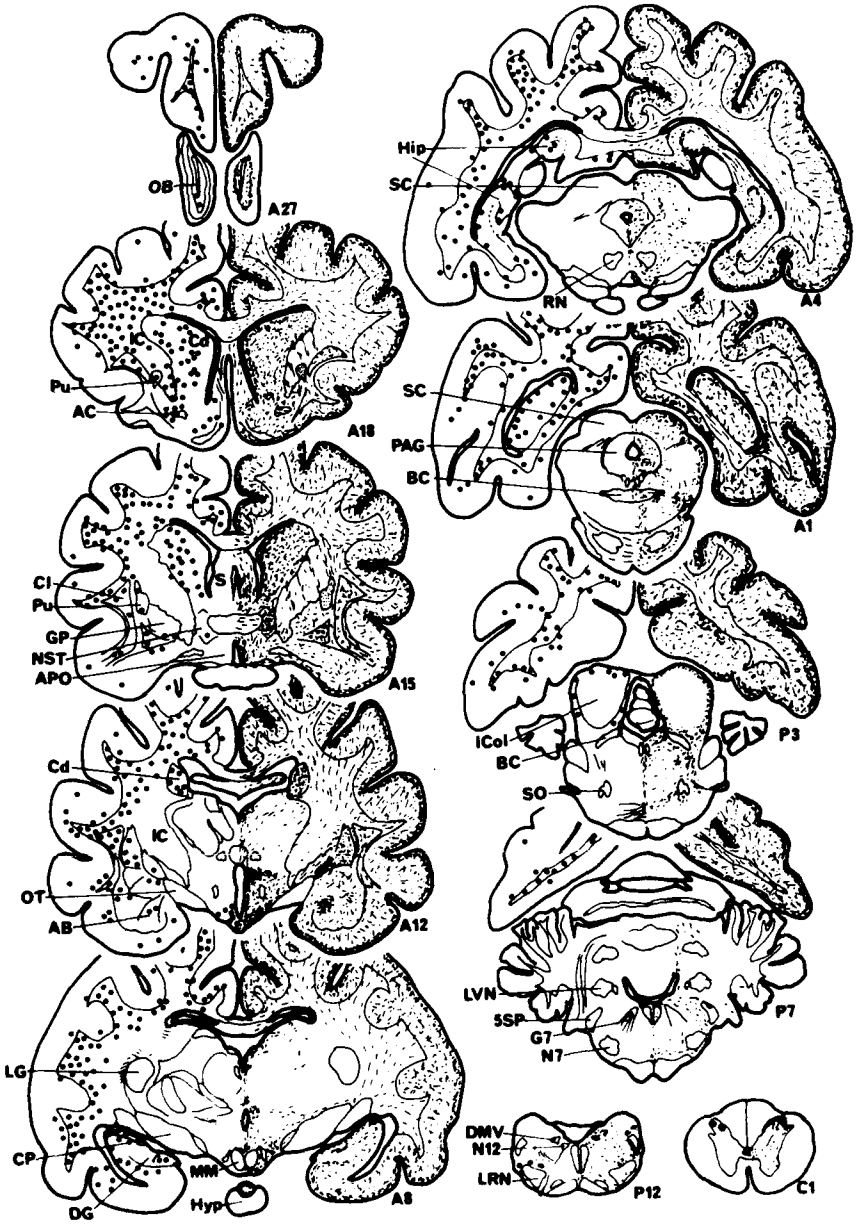

FIGURE 1. Schematic representation of the distribution of the NPY-immunoreactive cell bodies *(black dots on the left)* and fibers *(black lines on the right)* in the cat brain. Abbreviations : AB = amygdala, nucleus basalis; AC = anterior commissure; APO = preoptic area; BC = brachium conjuntivum; Cd = caudate nucleus; Cl = claustrum; CP = cerebral peduncle; DG = dentate gyrus; DMV = dorsal moto nucleus of the vagus; G7 = genu of the 7th nerve; GP = globus pallidus; Hip = hippocampus; Hyp = hypophysis; Icol = inferior colliculus; LG = lateral geniculate nucleus; LRN = lateral reticular nucleus; LVN = lateral vestibular nucleus; MM = medial mammillary nucleus; N7 = nucleus of the 7th nerve; N12 = nucleus of the 12th nerve; NST = bed nucleus of the stria terminalis; OB = olfactory bulb; OT = optic tract; PAG = periaqueductal gray; Pu = putamen; RN = red nucleus; S = septum; SC = superior colliculus; SO = superior olive; 5SP = spinal nucleus of the fifth nerve.

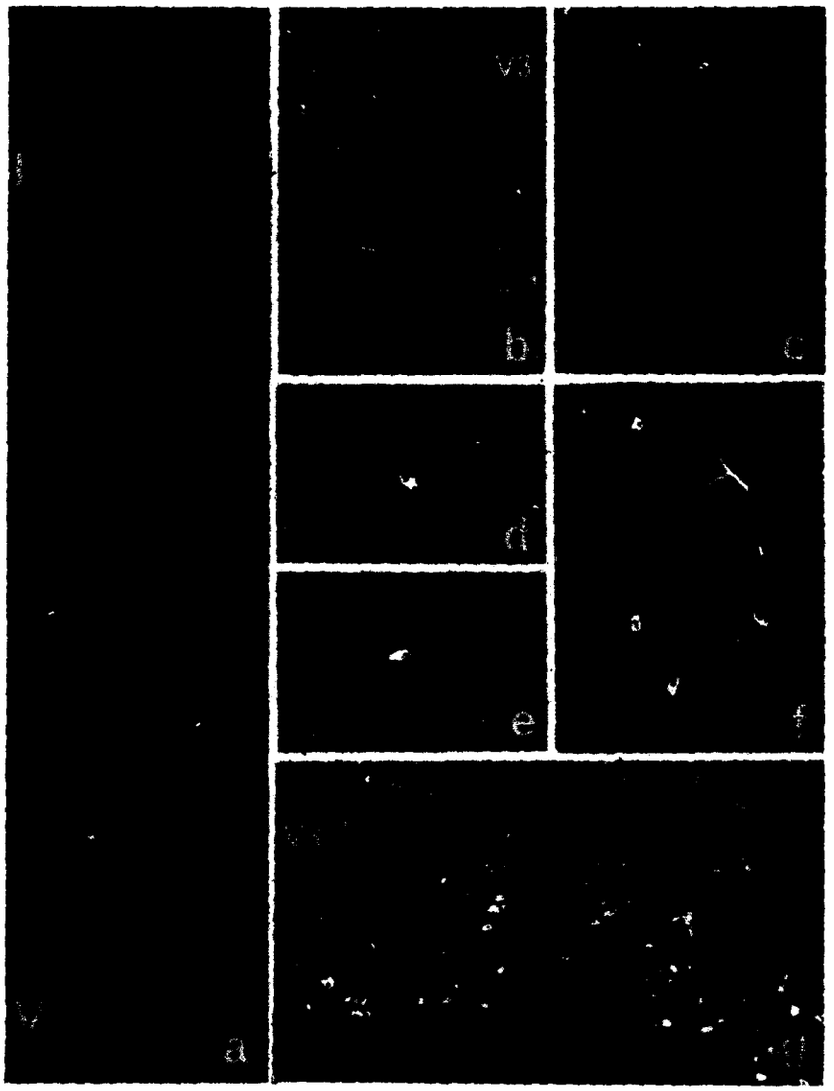

FIGURE 2. Illustrations of the NPY-immunoreactive neurons as seen with the immunofluorescence technique in the cat brain. **(a)** Immunoreactive axons through layers I to V in the cerebral cortex. *Arrows* point to labeled cell bodies; G × 120. **(b)** Paraventricular nucleus of the thalamus; G × 120. **(c)** Dorsal horn of the spinal cord. **(d)** and **(e)** Immunoreactive cell bodies in the cerebral cortex, at the border between white matter *(lower part of the figures)* and layers VI. Obliquely cut axons can be seen in the white matter; G × 120. **(f)** Caudate nucleus; G × 120. **(g)** Mediobasal hypothalamus; G × 30. V3: third ventricle.

neurons (FIG. 1). In the cortex, IR cell bodies were found throughout the white matter, in the deepest layers (FIG. 2a, d, e) and occasionally in the superficial layers. The innervation was in the form of long IR fibers running in the white matter and vertically across all layers in the gray matter (FIG. 2a) with denser plexuses in layer VI and under the pial surface. In the neostriatum, the caudate nucleus contained a large number of IR cell bodies lying among densely interwoven fibers (FIG. 2f), whereas the putamen displayed IR cells among fibers with mostly a terminal-like appearance. The amygdala received a dense NPY-IR fiber supply, especially the basal nucleus. In the hippocampus, IR cells were present in the hilus of the dentate gyrus and in or adjacent to the pyramidal layer. A dense plexus of fibers was observed in the stratum lacunosum moleculare.

In the hypothalamus, as noted previously,[15] a large population of IR cell bodies was visible in the mediobasal area after colchicine injection (FIG. 2g). These cell bodies were surrounded by a rich plexus of IR fibers, which were in continuity with abundant fibers in the periventricular, dorsal and ventrolateral areas. In the hypophysis numerous IR fibers passed along the pituitary stalk and ended in the neural lobe. The thalamus was poorly supplied with NPY-IR axons, except for the paraventricular nucleus (FIG. 2b). No IR cell bodies could be observed in this area of the brain.

In the brainstem, IR cell bodies were few compared to the rostral brain. Some were visible at the periphery of the inferior colliculus, in the pontine gray and the ventrolateral medulla. Moderately dense networks of IR fibers were seen in the periaqueductal and periventricular gray, the superior colliculus and periphery of the inferior colliculus, the lateral reticular formation, the dorsal pontine tegmentum and ventrolateral medulla. In the dorsal medulla, the nucleus of the tenth nerve and the dorsomedial part of the solitary tract nucleus contained dense aggregations of IR fibers. Patches of very dense IR plexuses in the spinal trigeminal root were continuous with abundant fibers in the superficial layers of the dorsal horn in the spinal cord (FIG. 2c).

The concentration of NPY in different areas of the rostral brain were measured by a double-antibody radioimmunoassay technique.[16,17] The displacement curves obtained with serial dilutions of acetic acid extracts of various brain regions were parallel to that of synthetic porcine NPY. The highest concentrations of NPY-like material were found in the dorsolateral hypothalamus, lateral geniculate and mediobasal hypothalamus (16.2 ± 3.0, 9.1 ± 3.6 and 6.6 ± 5.2 ng/mg protein, respectively). Reverse-phase high performance liquid chromatography (HPLC) analysis revealed that the NPY-IR material eluted as two major peaks which exhibited retention times shorter than synthetic porcine NPY.

Altogether, these data suggest that NPY located in the brain of the cat plays a variety of physiological actions. Since, most of the potential functions of NPY have been investigated in the rat, it will be of real interest to examine the behavioral and neuroendocrine effects of NPY in the cat.

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Localization and Characterization of Neuropeptide Y-Like Immunoreactive Peptides in the Nervous System and Midgut of *Locusta migratoria* and in the Brain of *Sarcophaga bullata*

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During the last few years an increasing number of investigations into insect neurohormones have been performed and it now appears that neuropeptides are involved in most insect regulatory processes. Several insect neuropeptides such as bombyxin, locustasulfakinin and leucosulfakinin, the locust diuretic hormone and the locustatachykinins exhibit sequence homologies with vertebrate hormones, insulin, gastrin/cholecystokinin, arginine-vasotocin and tachykinins respectively.[1] We have demonstrated the presence of substances related to the vertebrate 36 amino acid peptide NPY (neuropeptide tyrosine) in two insect species, *Locusta migratoria* and *Sarcophaga bullata*. NPY has a widespread distribution and is present in extremely high concentrations in the brain of vertebrates. The distribution of NPY-like substances in the nervous system and the midgut of the migratory locust, and in the brain of the fleshfly was determined by immunocytochemistry using an antiserum directed against synthetic porcine NPY. By means of the peroxidase-antiperoxidase method, NPY-immunoreactive perikarya were detected in the brain (FIG. 1), optic lobes, corpora cardiaca, suboesophageal ganglion and ventral nerve cord of the locust and in the brain, optic lobes and suboesophageal ganglion of the fleshfly.

The gross anatomic distribution of the NPY-immunopositive neurons and nerve fibers indicates that the NPY-like material can be regarded as a potential neurotransmitter or neuromodulator. The NPY-immunoreactive innervation of the locust gut musculature, in the same way as has been reported for the NPY-immunopositive nerve endings in the gut musculature of the guinea pig,[2] also points out a possible role of the NPY-like substance as an important neurochemical mediator in insects, *e.g.*, in the control of gut motility. NPY is known to have an inhibitory effect on the guinea pig ileal motility. Recently we isolated 12 neuropeptides from the locust brain which suppress the contractions of the insect gut.[3] These peptides will be sequenced in the near future. In the midgut many basal granulated cells which extend an apical process were intensively stained. Therefore, these NPY-immunoreactive cells, which are probably open to the gut lumen, might release their

secretions into the gut lumen. The presence of NPY-like material in the corpora cardiaca suggests that it also might be released into the haemolymph, serving as a neurohormone.

The concentrations of NPY-immunoreactive material in acetic acid extracts of locust brain, optic lobes, thoracic ganglia, ovaries and midguts were measured using a specific radioimmunoassay technique. The dilution curves of the crude tissue extracts were quite parallel to the NPY-standard curve. The highest amount of NPY immunoreactivity was found in the ovary (18 pg/mg tissue) and the midgut (4.6 pg/mg tissue). These findings confirmed and extended our immunohistochemical results and indicate that the nervous system and midgut of the locust contain a peptide related to NPY. This does not mean that both gut and nervous system contain the same immunoreactive material. In fact, reverse phase high performance liquid chromatography (RP-HPLC) in combination with the NPY-radioimmunoassay of a locust brain extract revealed the presence of three immunoreactive peaks (FIG. 2). The major peak (elution time 89 min) was less hydrophobic than the porcine NPY standard (elution time 100 min). The presence of two minor peaks (elution times 81 and 125 min) which were resolved under the same HPLC conditions may indicate the natural occurrence of two other distinct locust brain peptides which share amino acid homologies with NPY. Alternatively, they may result from chemical modifications of a single peptide during the extraction procedure. Such an heterogeneity of extracted NPY-like material has also been reported in different vertebrate species.[4] When compared to HPLC profiles of brain extracts, HPLC analysis of a gut extract revealed the presence of an additional major NPY-immunoreactive peak. This specific NPY-related gut peptide could represent another insect member of the pancreatic polypeptide family.

The widespread distribution of NPY-related peptides in the nervous tissue and in the gut suggests that they may have important functions in insects.

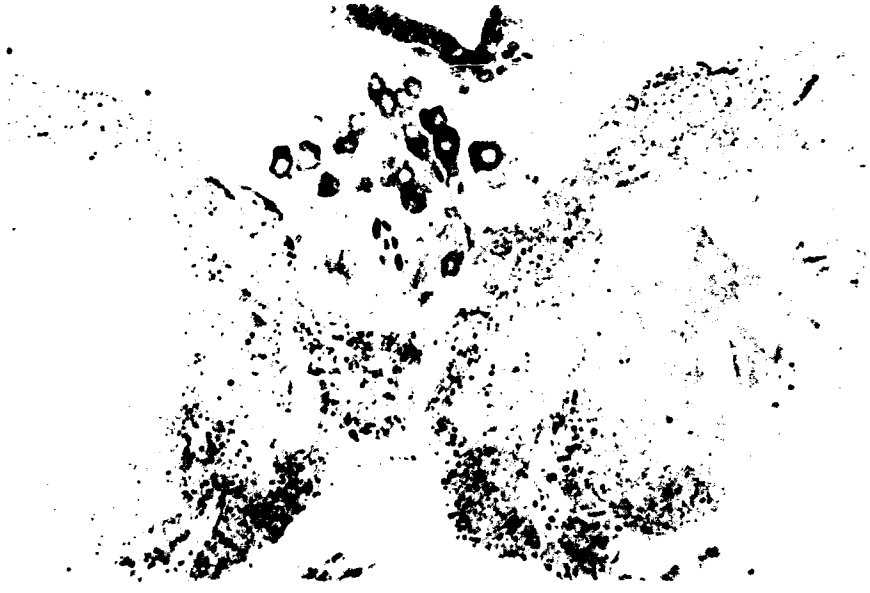

FIGURE 1. NPY-like immunoreactive cell bodies and nerve fibers in the brain of *Locusta migratoria*.

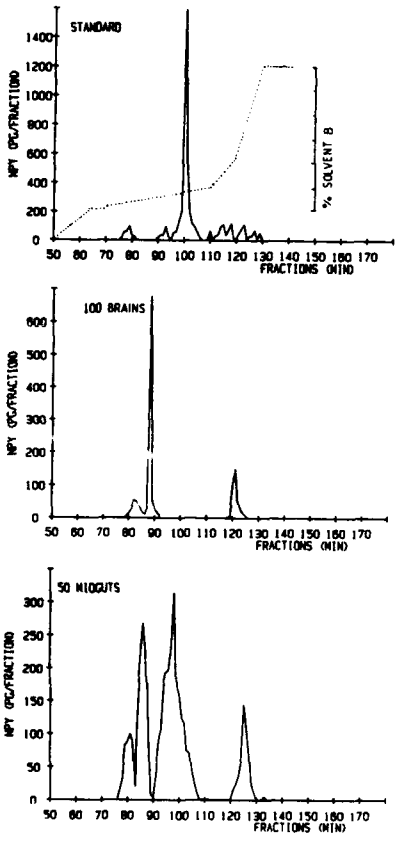

FIGURE 2. RP-HPLC and RIA determination of NPY-like immunoreactive peptides in the locust brain and midgut. Synthetic NPY and acid extracts of 100 locust brains and 50 midguts were applied to the Lichrosorb C18 column at a flow rate of 1 ml/min. The mobile phase consisted of 0.1% trifluoracetic acid/water (solvent A) and a gradient of and acetonitrile/methanol (80/20) mixture (solvent B). All one-minute fractions were assayed for NPY-like immunoreactivity.

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Electrophysiological Action of Neuropeptide Y on Cultured Frog Pituitary Melanotrophs[a]

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Phylogenic studies indicate that the structure of neuropeptide Y has been highly preserved during evolution.[1-5] In particular, in amphibians, biochemical and immunological approaches reveal the existence of an NPY-like peptide closely related to porcine or human NPY.[6-8] In the frog hypothalamus, NPY-producing neurons are located in the preoptic nucleus as well as in the dorsal and ventral infundibular nuclei.[6] A dense bundle of NPY-containing fibers innervates the intermediate lobe of the pituitary,[9] suggesting that NPY may be involved in the regulation of α-melanocyte-stimulating hormone (α-MSH) release from pars intermedia cells. Actually, synthetic porcine NPY causes a dose-related inhibition of α-MSH secretion from perifused neurointermediate lobes of frogs[9,10] and toads.[11] In order to understand the ionic events underlying NPY-evoked inhibition of hormonal release, the effects of NPY on membrane potential and major ionic currents were studied in cultured frog pituitary melanotrophs using the patch-clamp technique in the whole-cell configuration.

The recordings were conducted on 3- to 12-day old cells superfused with standard Ringer's solution (112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES, 11 mM glucose; pH adjusted to 7.4 with NaOH) as previously described.[12,13] The pipettes were filled with the intracellular solution (100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH adjusted to 7.4 with KOH). NPY (10^{-6} M) was administered for 3 to 10 s via pressure ejection from a glass pipette. In normal saline, among 22 cells tested at the resting potential, 18 responded to NPY application by a hyperpolarization varying in magnitude from 5 mV to 20 mV and persisting for 20–30 s. In spontaneously active cells the hyperpolarization was accompanied by an inhibition of the action potentials. When repeated hyperpolarizing pulses were applied, an increase of the membrane conductance was observed throughout the hyperpolarization induced by NPY. To further elucidate the ionic mechanisms which are responsible to the inhibitory action of NPY in melanotroph cells we have analyzed the effects of this neuropeptide on potassium and calcium currents. Voltage-dependent outward potassium current (IK_V), monitored by

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voltage steps from −60 mV to + 10 mV, repeatedly applied at 10-s intervals, was measured in conditions where sodium and calcium channels were blocked by tetrodotoxin (TTX, 10^{-6} M) and replacement of $CaCl_2$ with 2 mM $CoCl_2$. The intensity of IK_V evoked by constant depolarizing pulses dramatically increased when the cells were exposed to NPY (10^{-6} M) and returned to its initial value during rinsing with normal saline.

The effects of NPY on voltage-activated Ca^{2+} channels were also investigated using whole-cell current recordings, in conditions where calcium ions were the only carriers of inward currents. The electrodes were filled with a standard cesium-tetraethylammonium (TEA) solution containing 100 mM CsCl, 20 mM TEA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES, pH 7.4. The external solution contained TTX (10^{-6} M) and TEA (20 mM) with a high calcium concentration (10 mM). Two distinct voltage-dependent calcium currents, namely a nifedipine-sensitive sustained current (I_{CaS}) and a nifedipine-unsensitive inactivating current (I_{CaN}), previously identified in frog melanotrophs[12] were evoked by constant depolarizing pulses. Voltage steps from −80 mV to + 20 mV induced both I_{CaS} and I_{CaN}. The global current was reversibly reduced by NPY application. In the presence of nifedipine (10^{-5} M), the remaining current, I_{CaN}, completely disappeared when the cell was exposed to NPY, and the initial response was recovered during the washout. When the membrane potential was set at −40 mV, depolarizing steps to +20 mV elicited I_{CaS} alone. In all cells tested, this current was severely depressed by NPY application.

Taken together these data indicate that NPY-induced inhibition of α-MSH secretion in frog pituitary melanotrophs is associated with dramatic changes in the electrical activity of these cells. NPY causes hyperpolarization and inhibition of the spontaneous firing of cultured melanotrophs. The electrophysiological effects of NPY result from activation of voltage-gated potassium channels and blockage of voltage-operated S and N calcium channels.

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Interactions between Norepinephrine and Neuropeptide Y in Regulating Pancreatic Islet Hormone Secretion[a]

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Pancreatic islet hormone secretion is regulated through the combined action of nutrient, hormonal and neural secretagogues. Nerve fibers in pancreatic islets of a number of species contain both neurotransmitters and neuropeptides. We have recently demonstrated that peptides with HPLC and immunoreactive (IR) chracteristics identical to neuropeptide Y (NPY) are found in nerve fibers in anglerfish (AF) pancreatic islets.[1] In some species, NPY has been shown to affect basal or glucose-stimulated islet hormone release.[2-5] Since dopamine-β-hydroxylase-IR nerve fibers are found in AF islets, and NPY is often colocalized with NE in nerve terminals, we have investigated the possibility that NPY and NE interact in regulating basal or glucose-stimulated hormone secretion from AF islet cells.

Islet cells were dispersed using Dispase and cultured for a minimum of 48 hr in RPMI 1640 containing 2 mM glucose. Cells ($3-5 \times 10^6$) were suspended and perifused in an Endotronics APS 10 perifusion system linked to an Acusyst-S cell culture system and a multitube fraction collector. Basal incubation medium (IM) consisted of modified Krebs/Ringer buffer containing 2 mM glucose, 0.10% BSA and 15 mM Hepes. After 100 minutes of perifusion in basal IM, cells were perifused with IM containing KCl, glucose, NE and/or NPY. Challenges lasted 10 or 15 minutes depending on the experiment and up to four pulses of test substances were applied in a single experiment. Five-minute fractions were collected and aliquots taken for insulin, glucagon, and somatostatin RIA. Hormone release in each fraction was expressed as the % change ± SEM from baseline. Baseline was defined as the mean hormone release in the two fractions immediately preceding each pulse. The significance of changes in hormone release were determined using paired *t* tests to compare levels of hormone release in the fraction preceding pulse onset to levels of hormone release in the fraction following pulse onset.

Glucose stimulated both insulin and SS secretion in a dose-related manner with maximal increases of 3- to 4-fold occurring at 11 and 16.7 mM glucose, respectively. While 5.5 mM glucose stimulated glucagon secretion, higher concentrations were inhibitory. Application of NE (1 nM) stimulated insulin release 35.2 ± 9.3% ($p < 0.05$) while 10 nM NE had no effect. In contrast higher concentrations of NE (100 nM) inhibited insulin secretion ($-27.1 \pm 3.5\%$, $p < 0.005$). NE stimulated somatostatin secretion in a dose-related manner. Glucagon release was also stimulated by NE in a dose-related fashion; however in contrast to insulin and somatostatin the response pattern was biphasic.

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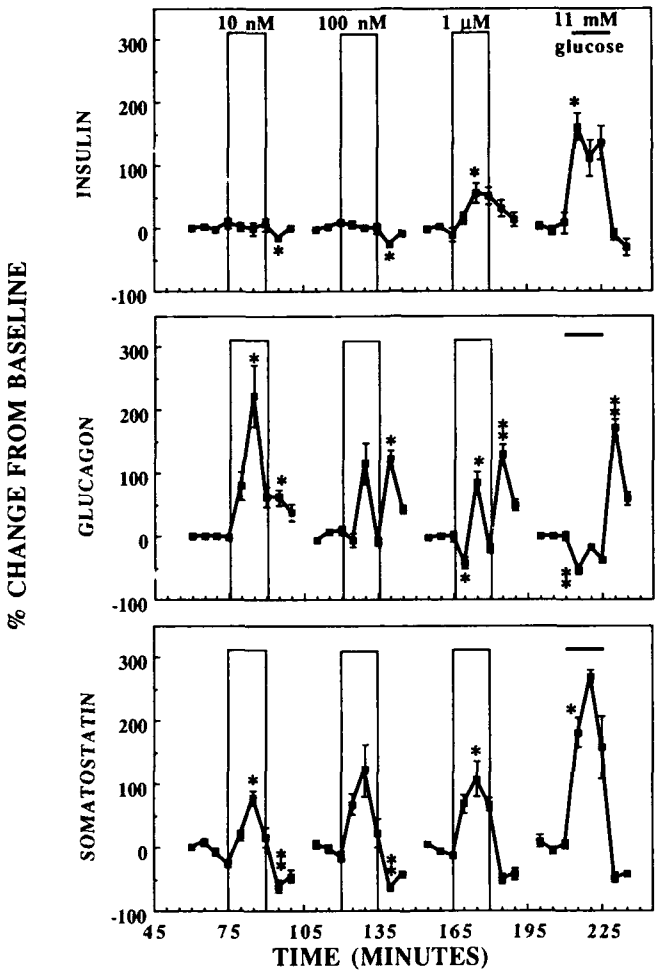

FIGURE 1. NPY, perifused in basal glucose conditions, modulates insulin, glucagon, and somatostatin secretion. Low concentrations of NPY (10 and 100 nM) had no effect on insulin secretion during the NPY pulse; however, both induced a significant postpulse inhibition of insulin secretion. The highest concentration of NPY (1 μM) stimulated insulin release 53 ± 14% and no postpulse inhibition was noted. NPY, at 10 nM, stimulated glucagon secretion 222.8 ± 48.5%. Higher concentrations of NPY induced a transient stimulation of glucagon release followed by a decrease to basal levels. NPY also induced a postpulse stimulation of glucagon secretion. In contrast to the NE response, this postpulse effect was not concentration dependent. As the NPY concentration increased, total glucagon secretion decreased in a dose-dependent manner. NPY (10 nM) stimulated somatostatin secretion 78.8 ± 10.7%. Higher NPY concentrations also increased somatostatin release; however, statistically significant differences in the magnitude of this effect were not observed. *$p < 0.05$, ** $p < 0.005$; paired t test with Bonferroni correction for multiple comparisons; n = 5.

NPY (1 μM) increased insulin release 53 ± 14% ($p < 0.05$). NPY stimulated SS secretion throughout the duration of the pulse whereas stimulatory effects on glucagon secretion were bimodal (FIG. 1). At 10 nM, NPY stimulated glucagon secretion 222.8 ± 48.5% ($p < 0.05$). Higher concentrations of NPY induced a transient stimulation of glucagon release followed by a decrease to basal levels. Furthermore, 1 μM NPY caused an initial inhibition of glucagon secretion followed by a transient stimulation. At both 100 nM and 1 μM NPY glucagon release was again stimulated after termination of the NPY pulse. When 10 nM NE and NPY were perifused together, NPY blocked the NE-induced stimulation of glucagon secretion in a dose dependent manner. Peak glucagon release decreased from 117 ± 34% ($p < 0.05$) when NE was applied alone, to 30.4 ± 18.4% when NE and 10 nM NPY were perifused together ($p < 0.05$) (FIG. 2). Interactions between NE and NPY in regulating insulin and SS secretion are currently being investigated.

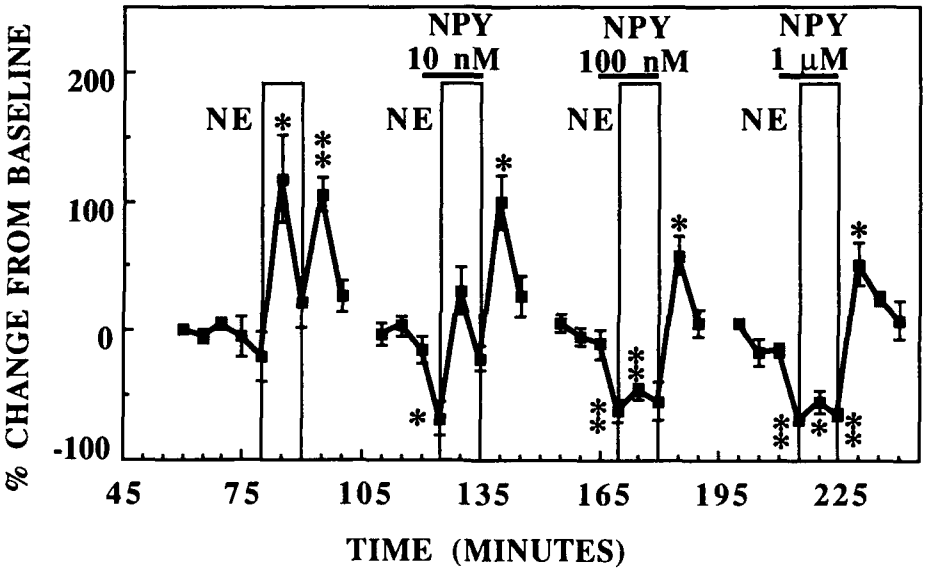

FIGURE 2. NPY interacts with NE to regulate glucagon secretion in basal glucose. NE (10 nM) or NE (10 nM) + NPY were applied at the times and concentrations indicated. NE stimulated glucagon release 120.4 ± 32%. Application of 10 nM NE + 10 nM NPY decreased peak glucagon release 4-fold to 30.4 ± 18.4% and increasing concentrations of NPY administered together with 10 nM NE decreased glucagon secretion below basal levels. Postpulse glucagon secretion in response to NE + 10 nM NPY did not differ from the postpulse response to NE alone. The postpulse response to higher NPY concentrations + 10 nM NE, differed from the postpulse response to NE alone in a statistically significant manner. (ANOVA, $p < 0.05$). * $p < 0.05$, ** $p < 0.005$; paired t test with Bonferroni correction for multiple comparisons; n = 5.

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Preproneuropeptide Y mRNA Expression in Glial Cell Cultures of the Neonate but Not 21-Day-Old Rat Brain

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INTRODUCTION

Neuropeptide Y (NPY) has been associated with the central and peripheral nervous systems since its isolation from porcine brain by Tatemoto in 1982.[1] Its function as a transmitter and modulator in the nervous system is supported by a large body of evidence. The expression of NPY in nonneuronal cells, however, has recently been reported.[2] For example, preproNPY mRNA has been identified in heart, spleen, adrenal gland, ovary, thymus, peripheral blood cells, bone marrow and lung.[3] With respect to these observations, it was of interest to determine if specific nonneuronal cell types present in the brain had the ability to express NPY. Therefore, preproNPY mRNA expression was examined in relatively pure cultures of astrocytes and oligodendrocytes prepared from rat brain at different stages of development.

METHODS

Neuron-enriched primary cultures from 1-day-old rat brains, astrocyte glial cultures from 1 and 21-day-old rat brains and oligodendrocyte cultures from 1-day-old rat brains were prepared as described previously.[4,5] These cultures contained more than 85% neurons, 98% astrocytes and 90% 02A-oligodendroctyes.

Total RNA was isolated from the various cell cultures by guanidinium thiocyanate extraction[6] and separated on agarose/formaldehyde gels by electrophoresis. PreproNPY mRNA was examined by Northern blot analysis utilizing a ^{32}P-labeled cDNA probe for NPY (generously provided by Dr. Janet Allen).[7] Hybridization conditions were: 50% formamide, 5 × SSC, 5 × Denhardts, 0.1% SDS, 42°C for 16–20 hr after a 3-hr prehybridization. Total ^{32}P-dCTP counts were quantitated by the Betascope Model 603 Blot Analyzer computer imaging system (Betagen Corp., Waltham, MA). Data are expressed as ^{32}P-dCTP counts minus background ^{32}P-dCTP counts. The Northern blot was exposed to Kodak XAR 5 X-ray film for 5 days after it was counted 18 hr on the Betascope Blot Analyzer, to produce an autoradiograph.

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RESULTS

Northern blot analysis detected a 0.8 kb preproNPY mRNA in neurons and astrocytes prepared from 1-day-old rats but not in astrocytes prepared from 21-day-old rats. Oligodendrocytes showed faint 0.8 kb bands in the 25 and 50 μg lanes which may be due to the presence of astrocytes (10% contamination) in these cultures (FIG. 1). Quantitation of this blot by computer image analysis indicated that the 25 and 50 μg neuronal RNA lanes had 563 and 1662 counts of ^{32}P-dCTP-NPY cDNA, respectively. Astrocytes prepared from 1-day-old rat brains contained 332 and 298 counts of ^{32}P-dCTP-NPY cDNA for the 25 and 50 μg RNA lanes, respectively (TABLE 1). The 25 and 50 μg lanes containing RNA from oligodendrocytes had 130 and 60 counts above background, respectively. The

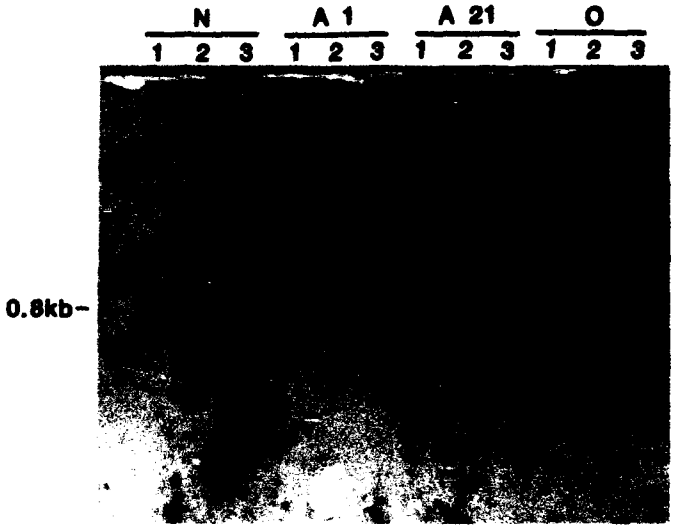

FIGURE 1. PreproNPY mRNA: autoradiograph of Northern blot of total RNA isolated from the various cell cultures and hybridized with ^{32}P-dCTP-labeled NPY cDNA. N = neuron enriched primary cells prepared from 1-day-old rat brains; A 1 = astrocytes prepared from 1-day-old rat brains; A 21 = astrocytes prepared from 21-day-old rat brains; and O = oligodendrocytes prepared from 1-day-old rat brains. 1 = 10 μg RNA, 2 = 25 μg RNA and 3 = 50 μg RNA.

neuronal cell cultures (28 counts/μg RNA) contained approximately 3 times more preproNPY mRNA than the astrocyte cell cultures (10 counts/μg RNA) prepared from 1-day-old rat brains. Furthermore, NPY was not detectable by RIA in pooled samples of oligodendrocytes, indicating that the preproNPY mRNA present in these samples was not translated appreciably.

DISCUSSION

These results indicate that preproNPY mRNA was present in astrocytes prepared from neonates but not 21-day-old rat brains. This age-related difference in expression suggests

TABLE 1. Preproneuropeptide Y mRNA

	^{32}P-dCTP-NPY cDNA Counts[a] Total RNA		
Cell Culture	10 μg	25 μg	50 μg
Neurons (1-day-old rats)	24	563	1662
Astrocytes (1-day-old-rats)	0	332	298
Astrocytes (21-day-old rats)	0	0	0
Oligodendrocytes (1-day-old rats)	0	130	60

[a]Values represent total number of ^{32}P-dCTP counts collected by a Betascope Blot Analyzer over an 18-hr period minus background. Average background (526 counts) was obtained from several arbitrary areas of the blot.

that preproNPY mRNA may be involved in brain development. Others have shown the presence of peptide mRNAs in cultured astrocytes in both a gene-and brain-region-specific manner.[8] For example, somatostatin mRNA was demonstrated in cerebellar but not neocortical or striatal astrocytes, while methionine enkephalin mRNA was present in astrocyte cultures prepared from all three brain regions.[8] Studies are underway to determine if the preproNPY mRNA present in neonate astrocytes is translated as well.

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Neuropeptide Y in Relation to Metabolism and Nutritional State

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INTRODUCTION

Neuropeptide Y (NPY) is widely distributed throughout the brain with particularly high concentrations in the hypothalamus[1] and, in particular, the paraventricular nucleus (PVN). NPY is one of the most potent stimulants of food intake, in particular carbohydrate intake, when injected into several hypothalamic sites, including the PVN.[2] In addition, PVN NPY injection has been shown to alter the release of CORT and insulin, both of which are known to be important in natural feeding processes.[1] Furthermore, content of NPY in the PVN has been shown to vary with the nutritional state of animals.[3,4] Additional evidence suggests the possibility that NPY may be involved in metabolic regulation.[5] In light of this evidence indicating that NPY may be involved in metabolic regulation, we have investigated the role of this peptide on such metabolic parameters as energy expenditure (EE) and respiratory quotient (RQ), as well as alterations in hypothalamic NPY levels as a function of nutritional state. This paper reviews these studies.

METHODS AND MATERIALS

In experiment 1, male Wistar rats were used for the determination of RQ and EE, after the administration of NPY directly into the PVN.

In experiment 2, three groups of male Sprague-Dawley rats were used. Group 1: satiated; Group 2: 48 hr food deprived; Group 3: 48 hr food deprived followed by 6 hr refeeding. The rats were sacrificed at the start of light period. The NPY content was measured by radioimmunoassay in 7 hypothalamic sites, namely PVN, supraoptic (SON), suprachiasmatic (SCN), dorsomedial (DMN), ventromedial (VMN) nuclei, perifornical lateral hypothalamus (LH) and arcuate nucleus-median eminence (ARC-ME).

RESULTS

Experiment 1

PVN NPY (39–156 pmol) significantly increased RQ, with the higher doses leading to longer latencies. Energy expenditure (EE) and activity remained unaffected by any dose.

Experiment 2

Forty-eight hr of food deprivation caused a 5-fold increase in NPY content in the PVN and a 10-fold increase in the ARC-ME. NPY levels were unchanged in all other hypothalamic sites tested. Six hr of refeeding was effective in restoring NPY levels in the ARC-ME but not in the PVN.

CONCLUSIONS

These results show that PVN NPY increases RQ, while having no effect on EE or activity levels. The NPY-induced increase in RQ suggests that NPY increases the catabolism of carbohydrate in favor of fat synthesis. This anabolic state, in which carbohydrate plays an important role, may explain the findings that acute PVN NPY injection selectively potentiates appetite for carbohydrates, specifically at the onset of the active cycle; and that chronic PVN NPY infusion has a potent effect on fat deposition and body weight and an increasing tendency to potentiate fat consumption.[2]

In addition to these effects on RQ, the results of the second experiment demonstrate that NPY levels in both the ARC-ME and PVN are sensitive to the nutritional status of the animal. The increase in PVN NPY content may reflect an increase in transport from the ARC-ME; alternatively, it may indicate a decrease in NPY release and catabolism within the PVN.

Taken together, these results suggest that PVN NPY may modulate both energy intake and energy substrate utilization. Since PVN NPY increases RQ, carbohydrate intake, and fat synthesis, all of which naturally occur at the onset of the active cycle, it is proposed that NPY in the PVN may play an important role in natural feeding specifically at that time of the diurnal cycle.

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Neuropeptide Y: Comparisons with Norepinephrine in Relation to Feeding Behavior

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Neuropeptide Y (NPY) is widely distributed in the brain with particularly high concentrations in the hypothalamus,[1] a structure considered to be critically involved in energy homeostasis and neuroendocrine/autonomic systems. This peptide coexists in certain hypothalamic neurons with the classical aminergic neurotransmitters, namely, norepinephrine (NE), epinephrine and serotonin (5-HT). Neuropeptide Y and NE are colocalized in the hypothalamic paraventricular nucleus (PVN) where administration of these substances stimulates feeding, in particular, carbohydrate intake in satiated rats.[2] The PVN is particulary rich in NPY-containing fibers as well as binding sites for this peptide. While several similarities between the effects of NPY and NE in the PVN suggest that this peptide and amine act through common neural substrates to stimulate feeding, other evidence indicates that their actions may be dissociable.

This report reviews the results of studies which used pharmacological and microdialysis/HPLC procedures to define the nature of the interaction between NPY and NE. The drugs used were the peptide, NPY, the monoamines, NE and 5-HT, the general α-receptor blocker phentolamine (PHT), the specific α_2-receptor blocker, rauwolscine (RAU) and the catecholamine (CA)-synthesis inhibitors, α-methyl-p-tyrosine (α-MpT) and Fla-63.

METHODS AND PROCEDURES

Subjects were adult male Sprague-Dawley rats with indwelling cannulae aimed at the PVN. The drugs used were administered directly into the PVN at a volume of 0.3 μl. Rats were given fresh food 1 hr prior to tests which were initiated by injecting one of the antagonists or vehicle followed by NPY, NE or their vehicles. Food intake was measured 1 hr postinjection. Animals in the microdialysis experiment had a 0.2 mm removable probe aimed at the PVN, and HPLC analysis of extracellular NE were conducted after local injection of NPY or vehicle.

RESULTS

The general α- and specific α_2-noradrenergic receptor blockers, PHT and RAU, respectively, failed to affect the feeding response induced by NPY. In contrast, RAU at the same doses significantly reduced NE-stimulated feeding ($p < 0.001$) causing a maximal suppression of approximately 85%.

The CA-synthesis inhibitors α-MpT and Fla-63 significantly enhanced, by over 100%, the eating responses elicited by PVN NPY administration ($p < 0.05$ for either α-MpT or Fla-63). α-Methyl-p-tyrosine did not affect the feeding response produced by exogenously administered NE.

The amine 5-HT failed to reduce NPY-induced feeding and it actually significantly enhanced this response ($p < 0.01$). In contrast, 5-HT attenuated NE-stimulated feeding ($p < 0.01$).

As measured by microdialysis, NPY tended to reduce extracellular levels of PVN NE, although this effect did not reach statistical significance. This is in contrast to the effect of galanin (GAL), another feeding stimulatory peptide that also coexists with NE in the PVN, which was found to cause a significant enhancement of NE levels.

CONCLUSIONS

The results of the present study show that the feeding response induced by NPY can be pharmacologically and biochemically dissociated from that induced by NE. In accordance with other studies, they suggest that NPY may act directly on its own peptide receptors, independently of postsynaptic α_2-receptors and presynaptic NE stores. In particular, our finding that reduced synthesis of NE results in a potentiation of NPY's feeding stimulatory action, in light of other evidence, suggests the existence of an antagonistic interaction between NPY and NE in the PVN.

Evidence indicates that the relative importance of NPY as a cotransmitter increases with the frequency of the nerve impulses.[3] That is, NPY coexists with NE primarily in large dense-cored vesicles, and intense, high frequency stimulation favors exocytosis of NPY from these large vesicles. This indicates that NPY exerts its effects on food intake specifically under conditions that evoke high frequency stimulation, such as when energy stores are most depleted by an extended period of little eating.

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Neuropeptide Y Effects on Feeding Behavior: Relationship to Norepinephrine and the Circadian System

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INTRODUCTION

Neuropeptide Y (NPY) is a potent stimulant of food intake when injected into the hypothalamic paraventricular nucleus (PVN).[1] In addition, NPY has been shown to affect many parameters important in natural feeding processes. These include: stimulatory effects on corticosterone (CORT) and insulin release,[2,3] both of which are known to increase food intake when injected systemically; suppressive effects on serotonin release,[4] which normally inhibits feeding; alterations in energy substrate utilization;[5] and effects on body weight regulation.[1] Furthermore, NPY levels in the PVN have been shown to fluctuate depending on the nutritional state of the animal.[6]

Macronutrient intake in the rat displays a circadian rhythm with carbohydrate intake beginning at the onset of the dark phase of the diurnal cycle, followed later in the dark by a decline in carbohydrate feeding with a corresponding increase in fat and protein intake.[7] The natural carbohydrate feeding at dark onset has been shown to require the presence of circulating CORT,[8] and the release of this hormone is affected by both NE and NPY.[2] This paper will review recent studies involving the effects of adrenalectomy (ADX) and CORT replacement on NE as well as NPY-induced feeding in relation to the circadian rhythm of macronutrient intake.

METHODS

Adult male Sprague-Dawley rats (n = 45) were stereotaxically implanted with chronic stainless steel guide cannulae aimed at the PVN. Following 3–5 days of recovery, 25 of the rats were randomly selected for either adrenalectomy (n = 15) or sham (n = 10) surgery. All rats were maintained on three pure macronutrient diets of protein (Casein, National Casein Co.; 3.7 Kcal/G), carbohydrate (a mixture of 30% dextrin, 30% corn starch, 40% sugar, ICN pharmaceuticals; 3.7 Kcal/G) and fat (lard, Armor; 7.7 Kcal/g). All diets were fortified with vitamins and minerals on a per-kilocalorie basis.

Injections of NPY (78 pmol), NE (40 nmol) or saline were given in counterbalanced order at different times of the light-dark cycle. Food intake was recorded 1 hr after injection. In CORT-replacement tests, CORT (Sigma; 0.5 mg/kg, in propylene glycol vehicle injected subcutaneously) was given ½ hr prior to PVN injection.

RESULTS AND DISCUSSION

After saline injection at the start of the dark period, rats displayed a preference for the carbohydrate diet (60% of total intake). In contrast, at the end of the dark carbohydrate

intake was minimal and rats displayed a preference for the protein (35%) and fat (43%) diets.

PVN NPY and NE injection elicited a strong feeding response at both the start and end of the dark period; however, the stimulatory effect on carbohydrate intake was stronger at the start as compared to the end of the night. The feeding response to PVN NE and NPY, regardless of injection time, was lost in ADX rats and restored after CORT replacement.

These results indicate that after injection into the PVN, both NE and NPY have their strongest stimulatory effects on carbohydrate intake at the start of the dark phase, when natural preference for this diet is at its peak. In addition, PVN NPY-induced feeding, like NE-induced feeding[9] and like natural carbohydrate feeding at dark onset,[8] is dependent on the presence of circulating CORT, which also reaches peak blood levels at this time of the cycle.

Recent studies,[5,10] indicate that both NE and NPY are anabolic in their actions, but have diverse effects on metabolism. While NE serves a conservative function, decreasing energy expenditure, while increasing food intake,[10] NPY enhances metabolic activity by increasing carbohydrate utilization and lipogenesis.[5] In addition, other results indicate that the feeding stimulatory and anabolic effects of NPY have a longer latency and duration that those of NE.[11] Based on these results, it is proposed that this peptide and amine may interact sequentially, specifically at the start of the dark phase in the process of initiating and maintaining the natural carbohydrate feeding chracteristic of the early dark period. At this time of the cycle, increases in circulating CORT, and PVN NE, may lead to the initiation of the initial carbohydrate meal. Subsequently, the utilization of NE, as well as increased CORT levels, may enhance the release or activity of NPY,[11] which through its inhibitory actions on serotonin release[4] and its excitatory actions on insulin[3] and CORT release,[2] may serve to prolong carbohydrate feeding at dark onset. NE and NPY may act cooperatively, albeit through different mechanisms, in their control of carbohydrate feeding and the restoration of energy balance particularly at the onset of the dark cycle.

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