REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		ANNUAL 15 Mar 93 TO 14 Mar 94

4. TITLE AND SUBTITLE

MOLECULAR APPRAOCH TO HYPOTHALAMIC RHYTHMS

5. FUNDING NUMBERS

F49620-92-J-0188

61102F

2312/CS

6. AUTHOR(S)

Dr J. Greagor Sutcliffe

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Scripps Research Institute
10666 North Torrey Pines Road
La Jollia, CA 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

AFOSR-TR- 94 0603

9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR/NL
110 DUNCAN AVE SUITE B115
BOLLING AFB DC 20332-0001

Dr Haddad

10. SPONSORING MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited

A

12b. DISTRIBUTION CODE

NTIS CRA&I	
DTIC TAB	
Unannounced	
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

13. ABSTRACT (Maximum 200 words)

The suprachiasmatic nucleus (SCN) of the hypothalamus is the anatomical seat of the mammalian endogenous biological clock which regulates the temporal expression of hormonal and behavioral circadian rhythms. Light, serotonin and melatonin are the dominant stimuli which affect the phase of the endogenous clock. The grantee has devised strategies to identify molecules that mediate the action of these stimuli within the SCN. The grantee has identified a novel receptor for serotonin, the 5-HT7 receptor, and determined its amino acid structure. Its pharmacological ligand binding properties have been measured and a unique profile of agonists and antagonists defined. These allowed demonstration that the 5-HT7 receptor mediated circadian activity of cultured SCN. The receptor has been shwon to couple to activation of adenylyl cyclase and to be synthesized by neurons of the subparaventricular zone immediately dorsal to the SCN. Molecules whose expression within the SCN is activated by light entraining cues have also been identified.

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(U)

I. SUMMARY:

The suprachiasmatic nucleus (SCN) of the hypothalamus is the anatomical seat of the mammalian endogenous biological clock which regulates the temporal expression of hormonal and behavioral circadian rhythms. Light, serotonin and melatonin are the dominant stimuli which affect the phase of the endogenous clock. The grantee has devised strategies to identify molecules that mediate the action of these stimuli within the SCN. The grantee has identified a novel receptor for serotonin, the 5-HT7 receptor, and determined its amino acid structure. Its pharmacological ligand binding properties have been measured and a unique profile of agonists and antagonists defined. These allowed demonstration that the 5-HT7 receptor mediated circadian activity of cultured SCN. The receptor has been shown to couple to activation of adenylyl cyclase and to be synthesized by neurons of the subparaventricular zone immediately dorsal to the SCN. Molecules whose expression within the SCN is activated by light entraining cues have also been identified.

II. OBJECTIVES:

The goal of the supported studies is to investigate the molecular mechanism that determine circadian rhythms by isolating cDNA clones of mRNAs expressed in the rodent suprachiasmatic nucleus that encode proteins involved in generating, regulating and responding to circadian environmental cues. In particular, studies are proposed to determine the receptors mediate entrainment by indoleamine neurotransmitters and to find the mRNAs whose expression is mediated by entraining cues, or that cycle circadianly within the SCN, or which are exclusive to the SCN.

III. STATUS:

A. Background

Serotonin applied during subjective day induces phase shifting of the circadian sleep/wake cycle in vivo as well as shifts in the circadian electrical activity of cultured suprachiasmatic nucleus (SCN) slices in vitro. The SCN is known from ablation and transplantation studies to be the brain organ which regulates circadian behaviors. The previous SCN slice work suggested that serotonin mediates the effect by elevation of cAMP concentrations and activation of protein kinase A. We therefore sought a 5-HT receptor associated with the SCN that was coupled to G_s.

We used a PCR-based method and nested pairs of primers corresponding to amino acid sequences conserved among the known (at that time 3) 5-HT receptors of the 7 transmembrane domain, G-coupled protein class, but which distinguished these receptors from non-serotonin receptors of the same class, to identify

clones of four novel putative serotonin receptors encoded by rat hypothalamic mRNAs. Each of the four ($5\text{-}HT_{1F}$, $5\text{-}HT_{5A}$, $5\text{-}HT_{5B}$ and $5\text{-}HT_7$) was shown by sequence and transfection studies to encode a protein of the 7 transmembrane domain receptor class to which, among physiological ligands, only serotonin bound with high affinity. The overall properties (anatomical distribution, pharmacology and physiological coupling) of the $5\text{-}HT_{1F}$, $5\text{-}HT_{5A}$ and $5\text{-}HT_{5B}$ receptors disqualified them for a primary involvement in circadian physiology.

B. The $5HT_7$ Receptor

The properties of $5\text{-}HT_7$ were, however, consistent with such a role. Northern blotting showed that its mRNA was expressed at its highest concentration in the hypothalamus. Membranes from transfected cells bound serotonin (and various agonists and antagonists) and transfected HeLa cells elevated cAMP concentrations in response to $5\text{-}HT_7$ and the agonists 5-CT and 8-OH-DPAT. This effect was antagonized by ritanserin, mesulergine and NAN-190, but not by the ß-adrenergic and $5\text{-}HT_{1A}$ antagonist pindolol. The primary sequence shared less than 40% identity with known 5-HT receptors.

C. Experimental Implication Of $5\text{-}HT_7$ In Circadian Rhythm

To test the hypothesis that $5\text{-}HT_7$ mediates the effect of serotonin in shifting the phase of circadian rhythms, and to rule out the prevailing alternative hypothesis that the effect was mediated by the $5\text{-}HT_{1A}$ receptor coupled unconventionally to a stimulatory G protein, we used the SCN slice preparation to assess the effects of a panel of agonists and antagonists that cumulatively could discriminate between these two alternatives as well as eliminate a role for all other known 5-HT receptors. In particular, we found a phase shift in neuronal electrical activity of SCN slices incubated with 5-HT, 5-CT and 8-OH-DPAT. The shift was blocked by cotreatment with ritanserin, but not pindolol. Ritanserin by itself has no effect. Among known 5-HT receptors, this pharmacological profile is exclusively consistent with $5\text{-}HT_7$.

D. Studies On The $5\text{-}HT_7$ mRNA

Northern blotting studies detected the rat $5\text{-}HT_7$ mRNA most prominently in the hypothalamus, at a lower concentration in the thalamus, and barely detectably in the cerebral cortex, hippocampus and medulla. At this level of detection, no $5\text{-}HT_7$ mRNA was observed in the cerebellum, striatum, heart, liver or kidney. By PCR analysis, the $5\text{-}HT_7$ mRNA could be detected in SCN punches and its concentration cycled with highest concentration from punches taken during the dark hours.

By in situ hybridization, thalamic expression was prominent in the anteroventral and paraventricular nuclei, expression

within the hippocampus was predominantly in the CA2 and CA3 pyramidal layers, and cortical expression was in piriform and retrosplenial areas as well as layers II and III of neocortex. Within the hypothalamus several areas were detected, including the anterior area and arcuate nucleus. Strong hybridization was observed in the subparaventricular zone of neurons which lie immediately dorsal to the SCN, receive approximately 75% of its projections, and send some of their projections back to the SCN.

E. Model For Role Of 5-HT In Circadian Regulation

The data suggest that the 5-HT_7 receptor is synthesized by subparaventricular zone neurons and cycles in concentration during the day. It is optimally available for binding serotonin at about noon. Binding activates G_s, which stimulates adenylyl cyclase to produce cAMP, which in turn activates PKA. The exact compartmental localization of the receptor within the neuron remains to be elucidated, as do the targets of PKA activation and how they effect a phase shift. Also, the physiological function of 5-HT_7 at its other sites of expression is unknown.

F. Isolation Of Human Homolog

The human homolog of 5-HT_7 has been isolated by PCR amplification from a human hypothalamus cDNA library. Progress was initially slow because available human libraries proved to be of low quality. We were unable to isolate clones with large inserts by direct screening or to amplify the entire 5-HT_7 open reading frame in a single step, probably because full-length clones are rarely or not represented in the libraries. The strategy which proved successful involved stepwise independent amplification of 5′ and 3′ fragments which overlapped at an internal XhoI site, followed by assembly of the cloned fragments into a baculovirus expression vector. During confirmation of the sequence, a one-base frame shift was detected 4 amino acids from the carboxy terminus. This has been repaired by synthesizing appropriate PCR primers. The final construct in the baculovirus vector is presently undergoing complete sequence analysis for final virus production. At that stage, stable transfected cell lines will also be produced in HeLa cells as a backup to the baculovirus expression clones. Membranes from infected/ transfected cells will be useful for identifying new ligands selective for 5-HT_7.

G. Immunological Studies

Six synthetic peptides corresponding to non overlapping regions within the rat 5-HT_7 amino acid sequence have been conjugated to an immunogenic carrier protein and used to elicit antisera in rabbits (2 rabbits/peptide). Sera have been collected at intervals post immunization and assayed for their

reactivity against the peptide antigens by ELISA: all are positive. A subset have been examined in a preliminary screen by sagittal section of rat brain, and two of these sera are promising in that they recognize neurons in the areas identified by in situ hybridization experiments.

Meanwhile several stably transfected HeLa cell lines expressing the rat 5-HT_7 receptor have been generated. These will be grown in the presence of radioactive amino acids and extracts will be used for immunoprecipitation with the antipeptide sera. Specificity will be determined by blocking with the appropriate peptides. Identity of the target will be assumed if there is coincident gel mobility with antisera to independent peptides that is consistent with the known 435 amino acid chain length of the receptor and its probable glycosylation. The latter will be verified by enzymatic deglycosylation. The antisera will then be used to examine immunoreactive structures within the SCN and subparaventricular zone of the hypothalamus, as well as other regions where the 5-HT_7 mRNA was detected. These studies will allow precise placement of 5-HT_7 within the neural circuitry that generates circadian rhythms, and may give insights into its roles at other sites of expression.

H. Generation of Knock-out mice

The gene encoding the mouse 5-HT_7 homolog has been isolated as a λ phage clone and its partial structure determined. A 6 kb fragment carrying the 0.8 kb Exon II (all ORF) 0.5 kb from its 5′ end has been isolated and a diphtheria toxin block ligated to the 5′ end of the fragment. The neo^r knockout cassette is presently being modified to produce appropriate restriction sites for insertion into the single KpnI site near the 5′ end of Exon II. The modified gene will then be transfected into embryonic stem cell line AB1, which along with a matched feeder cell line (SNL 76/7), is presently being cultured in the laboratory. Neo^r transfectants that escape diphtheria toxin killing will be assayed by PCR using a primer from within the neo cassette and one from the region of the 5-HT_7 gene upstream from that used in the knock-out construct so as to identify transformants in which 5-HT_7 has been inactivated by homologous recombination. These will be cultured and injected into mouse blastocysts for production of chimaeric heterozygotes, from which mice homozygous for 5-HT_7 null alleles will be bred.

The mice will be analyzed for their circadian activity patterns. The anatomical structure of their SCN regions will be examined. SCN slice cultures will be studies for their response to serotonin. Such studies will test the hypothesis that 5-HT_7 is the serotonin target that mediates circadian rhythms and may give specific insights into the circuitry involved. In addition, because 5-HT_7 expression is restricted to the limbic system, these mice may also shed light on 5-HT involvement in other

behavioral physiologies. The mice will be valuable for assessing the effects of putatively selective 5-HT_7 agonists and antagonists, both as models for drug administration, and also for comparing phenotypes of the mutants to physiological responses to the new drugs.

I. Cloning Of Differentially Expressed Genes Within Hamster SCN In Response To Light Entrainment

The SCN is the anatomical seat of the mammalian endogenous biological clock which regulates the temporal expression of hormonal and behavioral circadian rhythms. Previous studies indicate a strong correlation between the rapid and transient expression of mRNAs encoding immediate early genes (e.g., c-fos) and the time of day in which light is able to reset the endogenous pacemaker. Because most immediate early genes encode transcriptional factors, we speculate that a transcriptional cascade may be required for photic entrainment. Therefore, we have initiated a study to identify/clone differentially expressed mRNAs within the hamster SCN in response to photic entrainment. Hamsters were maintained under LD 14:10 for 14 days and then exposed at ZT19 to light for 15 minutes and then placed back into darkness. Micropunched SCNs of sacrificed hamsters at 30-60 minutes (labeled 30C = 30 min control; 30S = 30 min stimulated) and 180-225 minutes (180C and 180S) after light treatment were collected and poly(A) selected RNA extracted. To identify differentially expressed mRNAs a scheme devised for the construction of PCR-based cDNA libraries from small amounts of RNA (30-50 ng) was coupled to a newly developed subtractive hybridization procedure. The cDNA libraries appear representative of mRNA populations; concentrations of c-fos and NGFI-A cDNAs are induced ~10X in 30S vs. 30C and return to near baseline in 180C vs 180S whereas concentrations of c-jun and cyclophilin cDNAs remain unchanged among all four cDNA libraries. 97% of the mRNA mass which was in common between control and light-stimulated libraries was removed by subtractive hybridization. In our initial analysis, several cDNAs unique to 30S and 180S have been obtained and differential expression verified. These clones are presently being investigated in detail. This molecular approach has the potential of identifying the molecular substrate(s) by which light entrains the pacemaker cells within the SCN. Simultaneously, studies to identify mRNAs that cycle in or that are restricted to the SCN are proceeding by the same methodology.

IV. PUBLICATIONS:

1. Erlander, M.G., T.W. Lovenberg, B.M. Baron, L. deLecea and J.G. Sutcliffe (1993) Molecular approach to hypothalamic rhythms: Isolation of novel indoleamine receptor genes. J. Biol. Rhy. 8:25-31.

2. Erlander, M.G., T.W. Lovenberg, B.M. Baron, L. de Lecea, P.E. Danielson, M. Racke, A.L. Slone, B.W. Siegel, P.E. Foye, K. Cannon, J.E. Burns and J.G. Sutcliffe (1993) Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. Proc. Natl. Acad. Sci. USA 90:3452-3456.

3. Lovenberg, T.W., B.M. Baron, L. de Lecea, J.D. Miller, R.A. Prosser, M.A. Rea, P.E. Foye, M. Racke, A.L. Stone, B.W. Siegel, P.E. Danielson, J.G. Sutcliffe and M.G. Erlander (1993) A novel adenylate cyclase-activating serotonin receptor ($5\text{-}HT_7$) implicated in the regulation of mammalian circadian rhythms. Neuron 11:449-458.

4. Lovenberg, T.W., M.G. Erlander, B.M. Baron and J.G. Sutcliffe (1993) Cloning of new 5-HT receptors. International Clinical Psychopharmacology, Vol. 8, 19-23.

5. Danielson, P.E., J.B. Watson, D. Gerendasy, M.G. Erlander, T.W. Lovenberg, L. de Lecea, J.G. Sutcliffe and W.N. Frankel (1994) Chromosomal mapping of mouse genes expressed selectively within the central nervous system. Genomics 19:454-461.

V. PERSONNEL:

The following individuals have worked on the projects solely or partly supported by this grant

TSRI Scripps Research Institute

J. Gregor Sutcliffe, Ph.D.
Mark G. Erlander, Ph.D.
Timothy W. Lovenberg, Ph.D.
Guy Barry
Monica Carson, Ph.D.
Pamela E. Foye
Patria Danielson
Jeffrey E. Burns
Ana Dopazo, Ph.D.
Luis deLecea, Ph.D.
Keith Cannon

Other Collaborators

Bruce M. Baron, Ph.D.
Margaret Racke
Amy L. Slone
Barry W. Siegel
M.P. Cagle
R.L. Gannon
Joseph D. Miller, Ph.D.

Rebecca A. Prosser, Ph.D.
Michael A. Rea, Ph.D.

VI. INTERACTIONS:

Society for Research on Biological Rhythms, Amelia Island (Erlander)
Elba International Neuroscience Program, Marino di Campo, Italy (Sutcliffe)
World Congress on Psychiatric Genetics, New Orleans, (Sutcliffe)
Departments of Anatomy, Cell Biology and Physiology, UCLA (Sutcliffe)
Society for Neuroscience, Washington, DC (Sutcliffe, Erlander, deLecea, Dopazo)
American Society for Neurochemistry (Sutcliffe, Carson)
Northwestern University Medical School, Chicago (Sutcliffe)
University of South Dakota Medical School, Vermillion (Sutcliffe)
UC Irvine, Dept of Anatomy & Neurobiology, Irvine (Erlander)
Emory University, Dept of Pharmacology, Atlanta (Erlander)
Hershey Medical Center, Dept of Neuroscience & Anatomy, Hershey (Erlander)

VII. INVENTIONS:

None

Journal of Biological Rhythms, Vol. 8, Supplement, 1993, pp. S25–S31

Molecular Approach to Hypothalamic Rhythms: Isolation of Novel Indoleamine Receptor Genes

Mark G. Erlander,* Timothy W. Lovenberg,* Bruce M. Baron,† Luis de Lecea,* and J. Gregor Sutcliffe*

**Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037; †Marion Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215*

Abstract We have utilized polymerase chain reaction with primers corresponding to conserved amino acid sequences within membrane-spanning regions of known serotonin receptors to identify clones of four putative new indoleamine receptors. We have determined complete amino acid sequences of these four receptors, which fall into three subfamilies; two of these subfamilies are novel. The sites of expression within the brain have been determined for each of the genes. Expression in mammalian cells demonstrates that each new protein is a receptor for serotonin and that each has a distinct pharmacology when compared to known receptors. Two of the new receptors are coupled to cyclic adenosine monophosphate, one negatively (G_i) and one positively (G_s). The latter is a candidate for the serotonin receptor that mediates phase advances in circadian rhythms of the suprachiasmatic nucleus.

Key words 5-HT, serotonin, cAMP, G-protein-coupled receptor, cDNA cloning, PCR

The phases of endogenous circadian rhythms are modulated by the indoleamine neurohormonal transmitters serotonin (5-hydroxytryptamine, or 5-HT) and melatonin (Cassone, 1990; Krause and Dubocovich, 1990; Miller and Fuller, 1990; Medanic and Gillette, 1992; Edgar et al., 1993; Prosser et al., 1993). Biochemical studies indicate that the mechanism by which 5-HT and melatonin cause phase shifts in the suprachiasmatic circadian pacemaker is in part mediated by G-protein-associated receptors (Carlson et al., 1987; Medanic and Gillette, 1992; Prosser et al., 1993). Application of 5-HT to the *in vitro* suprachiasmatic nucleus (SCN) preparation (reviewed by Gillette, 1991) induces phase advances in spontaneous neuronal activity during subjective day; pharmacological studies indicate that 5-HT exerts this action via a 5-HT_{1A} or 5-HT_{1A}-like receptor (Medanic and Gillette, 1992; Prosser et al., 1993).

Genes encoding several subtypes of 5-HT receptors have been cloned, and except for 5-HT_3, all encode G-protein-associated molecules that span the plasma membrane seven times (Lubbert et al., 1987; Julius et al., 1988; Pritchett et al., 1988; Albert et al., 1990; Hamblin and Metcalf, 1991; Voigt et al., 1991; Hartig et al., 1992; McAllister et al., 1992). No gene encoding a melatonin receptor has yet been isolated; however, there is a melatonin receptor in the SCN (Vaněček, 1988), and melatonin binding to its receptor has been shown to mediate pertussis-toxin-sensitive inhibition of cyclic adenosine monophosphate (cAMP) accumulation (Carlson et al., 1989), suggesting that the receptor is a G-protein-coupled protein from the same protein superfamily. These data suggested to us that a study of

molecules expressed within the SCN that are related to 5-HT receptors might lead to identification of proteins involved in the mediation of indoleamine signaling in relation to rhythmicity, and also suggested a molecular strategy that would allow their identification.

STRATEGY

To isolate clones of indoleamine-binding receptors belonging to the superfamily of G-protein-associated receptors, we refined the strategy reported by Libert et al. (1989), who demonstrated that clones of novel members of this superfamily could be isolated through the use of degenerate primers corresponding to conserved regions of transmembrane domains (TMDs) III and VI in a polymerase chain reaction (PCR). We have extended this approach by performing two sequential rounds of nested PCR on a rat hypothalamic complementary DNA (cDNA) template (hypothalami were dissected during the middle of the subjective day and the middle of the subjective night in rats entrained to an LD 12:12 cycle), using degenerate primers in the second round of PCR that correspond to conserved residues specific to indoleamine-binding receptors. Our strategy increased the probability of isolating only indoleamine-binding receptors and amplifying indoleamine-binding receptors encoded by messenger RNAs (mRNAs) of low abundance, and decreased the probability of cloning artifactual cDNAs.

Two different variations of this general strategy were used to isolate clones of four novel indoleamine-binding receptors, designated as MR77, MR22, REC17, and REC20. The specific design that led to the subsequent cloning of MR77, MR22, and REC17 from hypothalamic cDNA was a first round of PCR using degenerate primers corresponding to conserved regions in TMDs III and VI of both catecholamine and 5-HT receptors. This was followed by a second round of PCR using a degenerate primer corresponding to a conserved region of TMD V, specific only to 5-HT receptors, in conjunction with the same TMD III primer used in the first round of PCR (Erlander et al., 1993; Lovenberg et al., 1993a).

For isolating REC20, we performed the first round of PCR-mediated hypothalamic cDNA amplification (here we used cDNA from rat cortex and striatum as well) using degenerate primers corresponding to regions of TMD III and TMD VII conserved among catecholamine and 5-HT receptors. The second PCR round used a degenerate primer corresponding to residues in TMD V specific to MR22 and REC17 in conjunction with the already used TMD VII primer (Lovenberg et al., 1993b).

SEQUENCE ANALYSIS

By performing these amplification strategies, we obtained PCR-derived cDNAs and used them as probes to screen a rat hypothalamus cDNA library (MR22, REC17, and REC20) or rat genomic library (MR77) to obtain clones spanning the entire protein-coding regions. We determined their nucleotide sequences and found that each putatively encoded a protein with the amino acid sequence characteristic of members of the G-protein-coupled, seven-TMD receptor superfamily. A search of the protein and nucleic acid data bases with the primary structures of these four putative receptors revealed that MR77 has significant amino acid sequence identity with known 5-HT receptors that are coupled to the inhibition of adenylate cyclase: human 5-HT_{1E} = 55%, dog 5-HT_{1D} = 48%, and rat 5-HT_{1B} = 46%. In contrast, MR22, REC17, and REC20 have 30–35% amino acid sequence identity with catecholamine and 5-HT receptors alike, with none exhibiting dominant similarity. However,

MR22 and REC17 have 68% mutual sequence identity. These observations suggest that MR77 is a member of the 5-HT_{1E} subfamily; that MR22 and REC17 form a new subfamily; and that REC20 falls into a subfamily by itself (Erlander et al., 1993; Lovenberg et al., 1993a,b).

mRNA DISTRIBUTIONS

We performed Northern blots with rat poly(A)-selected RNA extracted from eight brain regions (cortex, hypothalamus, thalamus, hippocampus, striatum, pons, medulla, and cerebellum) and from heart, liver, and kidney (Erlander et al., 1993; Lovenberg et al., 1993a,b). MR22 mRNA was detected only in the hippocampal sample. REC17 mRNAs were detected in hippocampus > cortex = thalamus = pons = striatum = medulla. REC20 mRNAs were found in hypothalamus = thalamus > pons = hippocampus. MR77 was not detected in any of the tissues examined by this method; however, by using a semiquantitative PCR technique, we found MR77 mRNAs in cortex = striatum = hippocampus > thalamus = pons > hypothalamus > cerebellum. None of the receptor mRNAs was detected in heart, liver, or kidney.

MR22, REC17, MR77, AND REC20 ARE ALL 5-HT RECEPTORS

To determine the ligand(s) for these four putative receptors empirically, we subcloned their cDNAs into a eucaryotic expression vector and transiently expressed the encoded protein in CosM6 cells. We found that broken cell preparations from transfected cells containing either MR22, REC17, MR77, or REC20 exhibited saturable binding for [^{125}I]LSD, whereas mock-transfected cells exhibited no measurable [^{125}I]LSD binding (Erlander et al., 1993; Lovenberg et al., 1993a,b). [^{125}I]LSD has been shown previously to be a nonselective 5-HT-ergic ligand with high affinity (K_D < 10 nM) for all known 5-HT receptors except 5-HT_{1B} (K_D = 10–1000 nM; Peroutka, 1990). The calculated equilibrium dissociation constant (K_D) for the individual receptors is as follows: MR22 = 2.0 nM, REC17 = 0.64 nM, MR77 = 14 nM, and REC20 = 1.5 nM. We next tested the ability of several biogenic amine neurotransmitters to displace [^{125}I]LSD binding from these receptors. 5-HT was able to displace [^{125}I]LSD binding to all receptors; the neurohormones melatonin, dopamine, and epinephrine had no measurable effects.

PHARMACOLOGICAL PROFILE AND CLASSIFICATION

To relate each of these receptors to known members of the 5-HT receptor family, we performed three analyses. We determined the ability of selective 5-HT agonists and antagonists to displace LSD from the membrane preparations. We compared their primary structures with other known 5-HT receptors by a dendrogram analysis (Fig. 1). We also expressed each receptor in HeLa cells to determine its ability to mediate cAMP accumulation.

[^{125}I]LSD binding to the MR77 protein is sensitive to sumatriptan, a 5-HT_{1D} agonist, but insensitive to 5-carboxyamidotryptamine (5-CT), a mixed 5-HT_{1A-1D} agonist, and 8-OH-DPAT, a 5-HT_{1A} agonist (Lovenberg et al., 1993a). The pharmacological profile of MR77 is similar to that of the 5-HT_{1E} receptor. Because MR77 has greatest identity (55%) with a recently cloned 5-HT_{1E} receptor, we conclude that we have identified a second member of the 5-HT_{1E} family and have designated MR77 as the 5-HT_{1F} receptor. This relation-

THIS
PAGE
IS
MISSING
IN
ORIGINAL
DOCUMENT

28 + 29

summarize what is known about MR77, MR22, REC17, and REC20. REC20 is a particularly good candidate for the receptor mediating 5-HT-induced phase advances in the SCN, given that Medanic and Gillette (1992) have demonstrated in an *in vitro* preparation of the SCN that a 5-HT-induced phase shift in electrical activity during subjective day is mediated by a 5-HT receptor that potently binds 8-OH-DPAT and 5-CT. In addition, these workers have shown that an increase in the cAMP concentrations during the same window of time during the subjective day results in a similar phase shift. REC20 is an appealing candidate for the relevant receptor, because it binds these 5-HT_{1A} agonists but, unlike $5HT_{1A}$, is coupled to increases in cAMP. Future studies are directed toward determining whether REC20 mRNA is present in the SCN (it is expressed in the hypothalamus, whereas 5-HT_{1A} mRNAs were undetectable in the SCN by *in situ* hybridization; Roca et al., 1[illegible]2), followed by use of pharmacological tools to discriminate functional responses mediated by REC20 and 5-HT_{1A} receptors within the *in vitro* SCN preparation.

ACKNOWLEDGMENTS

We thank our colleagues whose collaborative studies we have reviewed here. This work was supported in part by AFOSR Grant No. F49620-92-J-0188 and NIH Grant Nos. GM32355 and NS22347.

REFERENCES

ALBERT, P. R., Q.-Y. ZHOU, H. H. M. VAN TOL, J. R. BUNZOW, and O. CIVELLI (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine$_{1A}$ receptor gene. J. Biol. Chem. *265*: 5825–5832.

CARLSON, L. L., D. R. WEAVER, and S. M. REPPERT (1989) Melatonin signal transduction in hamster brain: Inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. Endocrinology *125*: 2670–2676.

CASSONE, V. M. (1990) Effects of melatonin on vertebrate circadian systems. Trends Neurosci. *13*: 457–464.

EDGAR, D. M., J. D. MILLER, R. A. PROSSER, R. R. DEAN, and W. C. DEMENT (1993) Serotonin and the mammalian circadian system: II. Phase-shifting rat behavioral rhythms with serotonergic agonists. J. Biol. Rhythms *8*: 17–31.

ERLANDER, M. G., T. W. LOVENBERG, B. M. BARON, L. DE LECEA, P. E. DANIELSON, M. RACKE, A. L. SLONE, B. W. SIEGEL, P. E. FOYE, K. CANNON, J. E. BURNS, and J. G. SUTCLIFFE (1993) Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. Proc. Natl. Acad. Sci. USA. *90*: 3452–3456.

GILLETTE, M. U. (1991) SCN electrophysiological *in vitro*: Rhythmic activity and endogenous clock properties. In *Suprachiasmatic Nucleus: The Mind's Clock*, D. C. Klein, R. Y. Moore, and S. M. Reppert, eds., pp. 125–143, Oxford University Press, New York.

HAMBLIN, M. W., and M. A. METCALF (1991) Primary structure and functional characterization of a human 5-HT_{1D}-type serotonin receptor. Mol. Pharmacol. *40*: 143–148.

HARTIG, P. R., T. A. BRANCHEK, and R. L. WEINSHANK (1992) A subfamily of 5-HT_{1D} receptor genes. Trends Pharmacol. Sci. *13*: 152–159.

JULIUS, D., A. B. MACDERMOTT, R. AXEL, and T. M. JESSELL (1988) Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science *241*: 558–564.

KRAUSE, D. N., and M. L. DUBOCOVICH (1990) Regulatory sites in the melatonin system of mammals. Trends Neurosci. *13*: 464–470.

LIBERT, F., M. PARMENTIER, A. LEFORT, C. DINSART, J. VAN SANDE, C. MAENHAUT, M.-J. SIMONS, J. E. DUMONT, and G. VASSART (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. Science *244*: 569–572.

LOVENBERG, T. W., M. G. ERLANDER, B. M. BARON, M. RACKE, A. L. SLONE, B. W. SIEGEL, C. M. CRAFT, J. E. BURNS, P. E. DANIELSON, and J. G. SUTCLIFFE (1993a) Molecular cloning and functional expression of 5-HT_{1E}-like rat and human receptor gene. Proc. Natl. Acad. Sci. USA. *90*: 2184–2188.

LOVENBERG, T. W., B. M. BARON, L. DE LECEA, J. D. MILLER, R. A. PROSSER, M. A. REA, P. E. FOYE, M. RACKE, A. L. SLONE, B. W. SIEGEL, P. E. DANIELSON, J. G. SUTCLIFFE, and M. G. ERLANDER (1993b) A novel adenylate cyclase-activating serotonin receptor (5-HT_7) implicated in the regulation of mammalian circadian rhythms. Neuron. In press.

LUBBERT, H., B. HOFFMAN, T. P. SNUTCH, T. VAN

DYKE, A. J. LEVINE, P. R. HARTIG, H. A. LESTER, and N. DAVIDSON (1987) cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of mRNA-injected *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA *84*: 4332–4336.

MCALLISTER, G., A. CHARLESWORTH, C. SNODIN, M. S. BEER, A. J. NOBLE, D. N. MIDDLEMISS, L. L. IVERSEN, and P. WHITING (1992) Molecular cloning of a serotonin receptor from human brain (5HT1E): A fifth 5HT1-like subtype. Proc. Natl. Acad. Sci. USA *89*: 5517–5521.

MEDANIC, M., and M. U. GILLETTE (1992) Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker *in vitro* only during the subjective day. J. Physiol. *450*: 629–642.

MILLER, J. D., and C. A. FULLER (1990) The response of suprachiasmatic neurons of the rat hypothalamus to photic and serotonergic stimulation. Brain Res. *515*: 155–162.

PEROUTKA, S. J. (1990) 5-Hydroxytryptamine receptor subtypes. Pharmacol. Toxicol. *67*: 373–383.

PRITCHETT, D. B., A. W. J. BACH, M. WOZNY, O. TALEB, R. DAL TOSO, J. C. SHIH, and P. H. SEEBURG (1988) Structural and functional expression of a cloned rat serotonin 5HT-2 receptor. EMBO J. *7*: 4135–4140.

PROSSER, R. A., R. R. DEAN, D. M. EDGAR, H. C. HELLER, and J. D. MILLER (1993) Serotonin and the mammalian circadian system: I. *In vitro* phase shifts by serotonergic agonists and antagonists. J. Biol. Rhythms *8*: 1–16.

ROCA, A. L., D. R. WEAVER, and S. M. REPPERT (1992) Serotonin receptor gene expression in the rat suprachiasmatic nucleus. Soc. Neurosci. Abstr. *18*: 1221.

VANĚČEK, J. (1988) Melatonin binding sites. J. Neurochem. *51*: 1436–1440.

VOIGT, M. M., D. J. LAURIE, P. H. SEEBURG, and A. BACH (1991) Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. EMBO J. *10*: 4017–4023.

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3452–3456, April 1993
Neurobiology

Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain

MARK G. ERLANDER*, TIMOTHY W. LOVENBERG*, BRUCE M. BARON†, LUIS DE LECEA*, PATRIA E. DANIELSON*, MARGARET RACKE†, AMY L. SLONE†, BARRY W. SIEGEL†, PAMELA E. FOYE*, KEITH CANNON*, JEFFREY E. BURNS*, AND J. GREGOR SUTCLIFFE*‡

*Department of Molecular Biology, MB10, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; and †Marion Merrell Dow Research Institute, Cincinnati, OH 45215

Communicated by Floyd E. Bloom, January 11, 1993

ABSTRACT **We report two serotonin (5-hydroxytryptamine, 5-HT) receptors, MR22 and REC17, that belong to the G-protein-associated receptor superfamily. MR22 and REC17 are 371 and 357 amino acids long, respectively, as deduced from nucleotide sequence and share 68% mutual amino acid identity and 30–35% identity with known catecholamine and 5-HT receptors. Saturable binding of ^{125}I-labeled (+)-lysergic acid diethylamide to transiently expressed MR22 in COS-M6 cells was inhibited by ergotamine > methiothepin > 5-carboxamidotryptamine > 5-HT. For REC17, the rank of potency was ergotamine > 5-carboxamidotryptamine > methiothepin > methysergide > 5-HT. Both were insensitive to 5-HT$_{1A}$, 5-HT$_{1D}$ or 5-HT$_2$ serotonergic ligands [8-hydroxy-2-(di-*n*-propylamino)tetralin, sumatriptan, and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane]. The mRNAs encoding MR22 were detected in the CA1 region of hippocampus, the medial habenula, and raphe nuclei. In contrast, mRNAs encoding REC17 were found throughout the rat central nervous system. We propose that REC17 and MR22, designated as 5-HT$_{5\alpha}$ and 5-HT$_{5\beta}$, represent a distinct subfamily of 5-HT receptors.**

Serotonin (5-hydroxytryptamine, 5-HT) regulates a wide variety of sensory, motor, and behavioral functions in the mammalian central nervous system. This biogenic amine neurotransmitter is synthesized by neurons in the raphe nuclei of the brainstem that project throughout the central nervous system, with the highest density in basal ganglia and limbic structures (1). Serotonergic transmission is thought to be involved with a variety of behaviors and psychiatric disorders including anxiety, sleep regulation, aggression, feeding, and depression (2, 3). Understanding how 5-HT mediates its diverse physiological actions requires the identification and isolation of the pertinent 5-HT receptors.

Molecular cloning has indicated that 5-HT receptors belong to at least two protein superfamilies: G-protein-associated receptors, which have seven putative transmembrane domains (TMDs) (5-HT$_{1A/B/C/D/E}$, 5-HT$_2$, and rat stomach fundus; refs. 4–18), and ligand-gated ion-channel receptors, which have four putative TMDs (5-HT$_3$; ref. 19). Libert *et al.* (20) demonstrated that novel G-protein-associated receptors could be identified by DNA polymerase chain reaction (PCR) amplification using degenerate primers corresponding to strongly conserved sequences within their TMDs. We have refined this strategy so as to target 5-HT-like receptors specifically. We report here the identification of a subfamily of 5-HT receptors whose members belong to the G-protein-associated superfamily.§

MATERIALS AND METHODS

PCR Cloning and Library Screening. PCR on poly(A)-enriched RNA from dissected rat hypothalami was performed exactly as described (21). The products were ligated into pBluescript (Stratagene) and used to transform *Escherichia coli* DH5α bacteria. Cloned DNA was used to probe an amplified rat hypothalamic cDNA library (4×10^6 recombinants in the unamplified library) that was constructed in λZAPII.

Anchored PCR. Poly(A)-enriched RNA from rat brain was converted to first-strand cDNA by using random hexamer oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase. The cDNAs were tailed with dGTP by terminal deoxynucleotidyltransferase. Anchored PCR (22) was performed using this dG-tailed cDNA as template. For MR22, a primer complementary to nt 385–404 (5′-GGATCCCATGCTTCTGCCGG-3′) of known MR22 sequence and an anchored primer (5′-GCACCGCGGAGCTCAAGCTTCCCCCCCCCCCCCCCCCC-3′) were used in an anchored PCR. Ten percent of the product was subjected to gel electrophoresis and transferred onto nitrocellulose, and the filter was hybridized with a radioactively labeled oligonucleotide representing nt 320–340 of the MR22 sequence. A major band of ≈400 bp was detected. The remaining product was digested with *Hin*dIII, the restriction fragments were separated by electrophoresis, and the 400-bp fragment was excised and subcloned into the *Hin*dIII site of pBluescript KS for nucleotide sequencing. For the REC17 anchored PCR, a primer complementary to nt 621–645 of REC17 was used and the radiolabeled REC17-specific probe was complementary to nt 557–571. A major band of ≈700 bp hybridized to the labeled probe and was excised, subcloned, and sequenced as described for MR22.

Expression in COS-M6 Cells. COS-M6 cells (subclone of COS-7) or HeLa cells were transfected with pDP5HT1a (23), pCMV4MR22 (MR22 cDNA), pCMV4REC17 (REC17 cDNA), or pBC12BIBeta2 (24) as described (21). Binding experiments using ^{125}I-labeled (+)-lysergic acid diethylamide (^{125}I-LSD) were performed exactly as reported (21).

RNA Blots. Total RNA was isolated from frozen tissues of adult Sprague–Dawley rats by extraction with guanidinium isothiocyanate (25). Oligo(dT)-cellulose chromatography (26) was used to enrich for poly(A)$^+$ RNA. For RNA blots, 10 μg of poly(A)$^+$ RNA was loaded per lane, except 1.3 μg for the medulla sample, and was subsequently resolved by electrophoresis in a 1.2% agarose/1.2 M formaldehyde gel, transferred to nitrocellulose membrane, and hybridized to either ^{32}P-labeled MR22 cDNA (entire insert) or the 3′ untranslated region of REC17 (nt 1200–1719). To confirm that similar amounts of intact RNA were loaded in each gel lane, blots

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); TMD, transmembrane domain; LSD, (+)-lysergic acid diethylamide.

‡To whom reprint requests should be addressed.

§The sequences reported in this paper have been deposited in the GenBank database [accession nos. L10072 (for MR22) and L10073 (for REC17)].

were stripped and hybridized with a ^{32}P-labeled cDNA probe for ubiquitously expressed cyclophilin mRNA (27).

***In Situ* Hybridization.** Free-floating *in situ* hybridization was performed as adapted from Gall and Isackson (28) and described by Lecea *et al.* (29). Coronal sections, 25 μm thick, from four young adult Sprague–Dawley rats were hybridized at 55°C for 16 hr with ^{35}S-labeled single-stranded RNA probes at 1.5×10^7 cpm/ml. Free-floating sections were then digested with RNase A at 4 μg/ml in 50 mM Tris·HCl/0.5 M NaCl/5 mM EDTA, pH 7.5, for 1 hr at 37°C. Washes were performed in 50% formamide/75 mM NaCl/7.5 mM sodium citrate, pH 7/14 mM 2-mercaptoethanol at 60°C for 3 hr and 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.5% sodium *N*-lauroylsarcosine at 68°C for 1 hr. Sections were mounted on coated slides, dehydrated, and exposed to Kodak XAR film for 5 days at room temperature. Autoradiography was performed by dipping slides in Ilford K4 emulsion diluted 1:1 with water and exposing them with dessicant at 4°C for 5 weeks. Slides were developed in Kodak D19, counterstained, and mounted in Permount.

RESULTS AND DISCUSSION

Strategy. We examined 5-HT receptors as a group to determine whether they contained amino acid sequences that distinguished them from other G-protein-coupled receptors. Sequences in TMD V received extra scrutiny because previous site-directed mutagenesis experiments of catecholamine receptors had demonstrated that this region is required for binding the catechol ring structure (reviewed in ref. 30). Our hypothesis was that the sequences required to bind indolamine ring structures might replace catechol-binding sequences in TMD V and that differences between indoleamine and catecholamine-binding sequences could be exploited experimentally to isolate indoleamine binders specifically. In the sequences of all 5-HT receptors available when we began this project (5-HT$_{1A}$, 5-HT$_{1C}$, and 5-HT$_2$), there was a consensus in TMD V that differed in two positions from catechol-binding receptors.

To test this hypothesis, cDNA was produced from rat hypothalamic mRNA and amplified in two sequential rounds of PCR. The primers in the first PCR round were degenerate, including all possible codons of the amino acid sequence L C A I A/S L D R Y in TMD III and the complement of C/M W L/C P F F I in TMD VI. This first round was expected to amplify sequences corresponding to most known catechol and all known 5-HT receptors. Amplified PCR products from this reaction were used as the substrate for a second round of PCR with the same 5′ primer and a 3′ primer that was a degenerate complement of all possible codons of F G/V A F F/Y I P L. This primer pair was expected to amplify sequences corresponding to 5-HT$_{1A}$, 5-HT$_{1C}$, and 5-HT$_2$ receptors and possibly those of novel 5-HT receptors. Adapter sequences on the 5′ ends of the primers allowed us to clone the doubly amplified cDNAs.

We used a pool of radiolabeled oligonucleotides corresponding to a nonconserved portion of the third intracellular loop for the rat 5-HT$_{1A}$ (nt 1002–976), 5-HT$_{1C}$ (nt 879–856), and 5-HT$_2$ (nt 1515–1488) receptors to screen $\approx 100^6$ bacterial colonies and thus eliminated clones corresponding to the known receptors from further consideration. Two clones, MR22 and MR77, failed to hybridize and were shown to have sequences distinct from one another. We report here analysis of MR22 and a related 5-HT receptor. MR77, a member of the 5-HT$_{1E}$ subfamily, is described elsewhere (21).

Identification of Two 5-HT Receptor-Like Proteins. We used the MR22 PCR-cDNA clone to screen 4×10^6 recombinants in an amplified rat hypothalamic cDNA library and obtained two clones that hybridized strongly and six clones that hybridized weakly.

The two strongly hybridizing clones had identical inserts of 1905 bp, containing an exact match to the original MR22 sequence; thus we continued the MR22 nomenclature. We performed anchored PCR with dG-tailed hypothalamic cDNAs as template and isolated an additional 319 bp. In composite, the MR22 cDNA is 2226 bp long and contains an open reading frame encoding 371 amino acids beginning 303 nt from the 5′ end (Fig. 1).

The six weakly hybridizing clones had identical inserts of 1511 bp. Because the nucleotide sequence was only 65% identical to that of MR22 and thus distinct, we gave this clone a different designation: REC17. Anchored PCR provided an additional 644 bp of sequence. In composite, the REC17 cDNA contains an open reading frame encoding 357 amino acids (Fig. 2). The amino acid sequences of the putative MR22 and REC17 proteins are 68% identical and can be optimally aligned with three gaps.

MR22 and REC17 Form a Subfamily of G-Protein-Associated Receptors. Both MR22 and REC17 contain seven putative membrane-spanning regions, as well as putative N-glycosylation and phosphorylation sites in positions similar to those found in known receptors (Figs. 1 and 2), consistent with the hypothesis that these two proteins belong to the G-protein-associated receptor superfamily. A search of the GenBank 74.0 and Swiss-Prot 23.0 databases (December 1992) with the entire MR22 putative protein sequence revealed the greatest identities, in the 30–35% range, to α- and β-adrenergic and 5-HT receptors from various species. REC17 yielded very similar results. Comparison of only the putative TMD regions of MR22 and REC17 with TMDs of

```
GGTTCCCAGTGTGCAGGCATCAGTCCCCAGTTCTGCAGGCGGTTGGTTACTCTGAAGACC      60
ACAAAGAGACTGGGAGAGGTTGATGCGCTGGACAAAGCTAGACTAAGGAGTCTCAACTGG     120
AAAAAAGGGTCTACGAAAACCTCAAAAAAGAAGCGCCTACAGTTTGGAAAAAGAACAAAG     180
GTGGCGCGGCTTAGACTTCTTTTTGTGTTGCTGGGCTCGCGCAGTGCCCCTCCTGCCTCG     240
CCACCTAACCACAGTTCATGCAATCACGGGCACATCTGCCAGAGAGCCCGAGTCCCTGAA     300
CAATGGAAGTCTCTAACCTCTCAGGCGCCACCCCTGGCATTGCCTTTCCTCCGGGACCCG     360
  M  E  V  S  N  L  S  G  A  T  P  G  I  A  F  P  P  G  P  E      20
AGAGCTGCAGTGACAGCCCAAGTTCCGGCAGAAGCATGGGATCCACCCCAGGGGGGCTCA     420
  S  C  S  D  S  P  S  S  G  R  S  M  G  S  T  P  G  G  L  I      40
TCTTGTCCGGCCGCGAGCCGCCCTTCTCTGCCTTCACCGTACTCGTGGTAACTCTACTGG     480
  L  S  G  R  E  P  P  F  S  A  F  T  V  L  V  T  L  L  V      60
TGTTGCTGATCGCTGCCACTTTCTTATGGAATCTGCTAGTTCTGGTGACTATCCTGCGCG     540
  L  L  I  A  A  T  F  L  W  N  L  L  V  L  V  T  I  L  R  V      80
TCCGCGCCTTCCACCGTGTGCCACATAACTTGGTAGCCTCGACCGCCGTCTCGGACGTCC     600
  R  A  F  H  R  V  P  H  N  L  V  A  S  T  A  V  S  D  V  L     100
TGGTGGCGGCTCTGGTGATGCCACTGAGCCTGGTGAGCGAGTTGTCGGCTGGGCGACGTT     660
  V  A  A  L  V  M  P  L  S  L  V  S  E  L  S  A  G  R  R  W     120
GGCAGCTGGGCAGGAGTCTGTGCCACGTGTGGATCTCCTTCGACGTGTTGTGCTGCACAG     720
  Q  L  G  R  S  L  C  H  V  W  I  S  F  D  V  L  C  C  T  A     140
CCAGCATCTGGAACGTGGCGGCCATCGCCCTGGATCGCTACTGGACTATCACGCGCCACC     780
  S  I  W  N  V  A  A  I  A  L  D  R  Y  W  T  I  T  R  H  L     160
TGCAGTACACGCTGCGCACCCGGCGCCGCGCTTCAGCACTTATGATCGCGATCACCTGGG     840
  Q  Y  T  L  R  T  R  R  R  A  S  A  L  M  I  A  I  T  W  A     180
CACTGTCCGCCCTCATCGCGCTCGCCCCGCTGCTCTTTGGCTGGGGCGAAGCCTACGATG     900
  L  S  A  L  I  A  L  A  P  L  L  F  G  W  G  E  A  Y  D  A     200
CTCGGCTGCAGCGTTGCCAGGTGAGCCAGGAGCCTTCGTACGCCGTCTTCTCCACCTGCG     960
  R  L  Q  R  C  Q  V  S  Q  E  P  S  Y  A  V  F  S  T  C  G     220
GAGCTTTCTACGTGCCTCTGGCCGTGGTGCTCTTCGTCTACTGGAAGATATACAAAGCCG    1020
  A  F  Y  V  P  L  A  V  V  L  F  V  Y  W  K  I  Y  K  A  A     240
CCAAGTTTCGATTCGGCCGCAGACGGCGGGCGGTAGTGCCCCTGCCCGCCACCACGCAGG    1080
  K  F  R  F  G  R  R  R  R  A  V  V  P  L  P  A  T  T  Q  A     260
CAAAGGAAGCACCTCAGGAGTCTGAGACGGTATTCACCGCGCGTTGCAGAGCGACAGTGG    1140
  K  E  A  P  Q  E  S  E  T  V  F  T  A  R  C  R  A  T  V  A     280
CCTTCCAGACAAGTGGAGACTCCTGGCGGGAGCAGAAGGAGAAGCGAGCCGCCATGATGG    1200
  F  Q  T  G  D  S  W  R  E  Q  K  E  R  R  A  A  M  M  V     300
TGGGGATCTTGATCGGTGTGTTTGTGCTGTGCTGGATCCCCTTCTTCCTGACGGAGCTCG    1260
  G  I  L  I  G  V  F  V  L  C  W  I  P  F  F  L  T  E  L  V     320
TCAGCCCGCTCTGCGCCTGCAGCCTGCCACCCATCTGGAAAAGCATATTCCTGTGGCTTG    1320
  S  P  L  C  A  C  S  L  P  P  I  W  K  S  I  F  L  W  L  G     340
GCTATTCAAATTCGTTCTTCAATCCCTTAATCTACACGGCCTTTAATAAGAACTACAACA    1380
  Y  S  N  S  F  F  N  P  L  I  Y  T  A  F  N  K  N  Y  N  N     360
ATGCCTTCAAGAGCCTCTTTACTAAGCAGAGATAAGCAGGGCTGGGGAGATAAAAAGGAA    1440
  A  F  K  S  L  F  T  K  Q  R  *                               370
GACCGGGGAAGAGAAAAGGGGATCTGCCGTCCTCATTTCACCAGAGACCTGGGGGCTTCTC    1500
CCCGCCGCCCACACCCCCCTAACGACACTCCAGAAATCACACCGTAGGGCCTGGAATGTT    1560
GAGTTCTCACGAAGGTAGACAACGGTTTGGCAAATACATCCATGCCTTCTACGCGTCGTG    1620
ACAGACATTGCTAGTGAATTGTGCTACATTTCTGCACCAGGCAGGAACCCCGCCAAACCC    1680
TTTCCGGGTGTATTTTAGGTATTATTGCTCATTTGTCAGACTCCACATGTGGCAGAGTTG    1740
ATTTGCTTTTGCGTTAGTACTATGTCCCTCAGTGAGGGTCTCTTGGGGCTCTCCTGAGAC    1800
TGACTCCTTGACATAGCCTCTCCTCTACCCCTTATCCATCAGAGCACTTTACCTTTCTTA    1860
GCCTCACACAGGACCTCCACAAGGCAATGATTCTCAGTTTAGGAAGAGATGTCCTCGCCT    1920
GAAGCTTTGTTAAAAAGTGTTCCACACAGACGTCGTCAAGATGGCTCAGTAGAGGGGGTT    1980
GGGGATTTAGCTCAGTGGTAGAGCCCTTGCCTAGGGAGCGCAAGGCCCTGGGTTCGGTCC    2040
CCAGCTCCGAAAAAAAGAACCAAAAAAAAAAAAAAAAAAAAAAAAAAAGATGGCTCAGTAGA    2100
TGAAGGCGCCTGTCCCCAAGCCTGGTGGCCTGCTTTTGAGATACATGTAATGGAAGGAAA    2160
TAAAATGATTGCAAGTTGTCTCTGACCTCCAGATATGTGCCATCAGCCCTCTCCCCCATG    2220
TGCACA                                                          2226
```

FIG. 1. Nucleotide sequence and predicted amino acid sequence of the rat MR22 cDNA clone. Putative TMDs are underlined, a putative N-glycosylation site is circled, and putative protein kinase C phosphorylation sites are boxed.

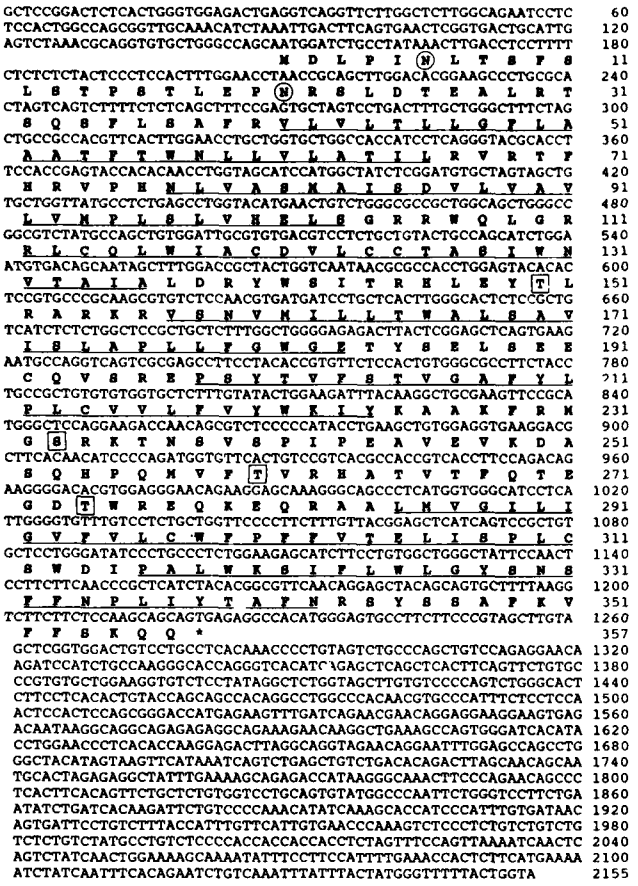

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the rat REC17 cDNA clone. Putative TMDs are underlined, two putative N-glycosylation sites are circled, and putative protein kinase C phosphorylation sites are boxed.

other known G-protein-associated receptors gave qualitatively similar results, although the identities were in the 40–48% range, with none exhibiting dominant similarity. Thus, the MR22 and REC17 proteins are probable G-protein-associated receptors that are much more related to one another than to known receptors for various ligands. Consequently, sequence analysis does not allow us to postulate the identity of the endogenous ligands interacting with MR22 and REC17.

MR22 and REC17 Are 5-HT Receptors. To determine the ligand(s) for MR22 and REC17 empirically, we subcloned the two cDNAs into a eukaryotic expression vector (pCMV4) and transiently transfected COS-M6 cells. We found that broken-cell preparations from transfected cells containing either MR22 or REC17 exhibited saturable binding for ^{125}I-LSD, a nonselective serotonergic ligand. For MR22, the calculated equilibrium dissociation constant (K_D) was 4.8 nM, while for REC17 it was 1.7 nM. To compare these values with those of known 5-HT receptors, we measured the saturation binding of ^{125}I-LSD to membranes prepared from 3T3 mouse fibroblasts stably expressing the rat 5-HT$_{1C}$ and 5-HT$_2$ receptors. Our values, 0.72 nM for 5-HT$_{1C}$ and 5.2 nM for 5-HT$_2$, are in relative agreement with previously reported values (2.5 nM for 5-HT$_{1C}$ and 12.5 nM for 5-HT$_2$; ref. 31).

We tested the ability of several cationic neurotransmitters to inhibit binding of ^{125}I-LSD to these receptors (Table 1). 5-HT at 380 nM competed for half (IC_{50}) of ^{125}I-LSD binding to REC17; the calculated K_i is 239 nM. For MR22, the IC_{50} for 5-HT was considerably higher, 1613 nM (K_i is 1333 nM). We measured the competition binding of ^{125}I-LSD to membranes prepared from 3T3 fibroblasts stably expressing the rat 5-HT$_{1C}$ and 5-HT$_2$ receptors and obtained similar K_i values, 92 nM and 1021 nM, respectively. Neither dopamine, norepinephrine, nor melatonin at ≤ 1 μM inhibited ^{125}I-LSD binding to REC17 or MR22 detectably.

Table 1. Competition for ^{125}I-LSD binding sites on MR22- or REC17-transfected COS-M6 cell membranes

Ligand	K_i, nM	
	MR22	REC17
Ergotamine	15.7	6.3
5-CT*	235.5	12.6
Methiothepin	145.5	28.9
5-HT	1333	239.0
Methysergide	>1000	195.6

COS-M6 cells were transfected with expression vectors containing either MR22 or REC17 cDNAs. Cell membranes were labeled with ^{125}I-LSD, which was competitively antagonized by a variety of ligands. IC_{50} values were used to calculate K_i values according to the equation (32) $K_i = IC_{50}(1 + C/K_D)$, where IC_{50} values represent the concentrations of ligands (nM) at which 50% of the bound ^{125}I-LSD could be displaced, C is the ^{125}I-LSD concentration (1 nM), and K_D is the equilibrium dissociation constant of ^{125}I-LSD (4.8 nM for MR22; 1.7 nM for REC17). The following compounds were ineffective ($K_i > 1000$ nM) in inhibiting ^{125}I-LSD binding to both MR22 or REC17: methysergide, yohimbine, metergoline, SKF 83566, 8-hydroxy-2-(di-*n*-propylamino)tetralin, mesulergine, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, zacopride, dopamine, norepinephrine, and melatonin.
*5-Carboxamidotryptamine.

The affinity of representative serotonergic receptor ligands was determined by their ability to compete for the specific binding of ^{125}I-LSD (Table 1). MR22 and REC17 generally had similar, though not identical, pharmacological profiles. Ergot alkaloids, and in particular, ergotamine, were the most potent ligands. However, within this compound class there were some notable differences. Thus, methysergide exhibited at least 5-fold selectivity for REC17, whereas neither receptor recognized metergoline or mesulergine. Although both MR22 and REC17 bound 5-carboxamidotryptamine with nigh affinity, the agonist binding profile of these receptors was easily differentiated from that of the 5-HT$_{1D}$ and 5-HT$_{1A}$ receptors in that both MR22 and REC17 were insensitive to sumatriptan and yohimbine (5-HT$_{1D}$ ligands) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (5-HT$_{1A}$ ligand). Finally, 5-HT$_2$-selective [1-2,5-dimethoxy-4-iodophenyl)-2-aminopropane and SKF 83,566] and 5-HT$_3$/5-HT$_4$-selective (racemic zacopride) selective compounds were ineffective.

To determine the effector-coupling mechanisms of MR22 and REC17, we tested whether MR22 and REC17 could mediate the inhibition or activation of adenylate cyclase by serotonin. In HeLa or COS-M6 cells cotransfected with either MR22 or REC17 and the hamster β_2-adrenergic receptor, we found no reduction in isoproterenol-stimulated cAMP accumulation (data not shown). As positive controls, we cotransfected cells with the rat 5-HT$_{1A}$ receptor or MR77 (21) and found significant reductions in isoproterenol-stimulated cAMP accumulation (55% and 32%, respectively; data not shown). Neither MR22 nor REC17 would stimulate the accumulation of cAMP in either cell line (data not shown). These results suggest at least three possibilities: (*i*) MR22 and REC17 couple to a second messenger other than cAMP, (*ii*) the G protein necessary for efficient coupling is missing from the cells assayed, or (*iii*) our transient transfection assay lacks requisite sensitivity.

mRNAs Encoding MR22 and REC17 Are Differentially Expressed in Rat Brain. We hybridized MR22 and REC17 cDNAs to Northern blots of RNA from rat brain regions and heart, liver, and kidney. Detection of RNAs with either cDNA required 2–3 weeks of autoradiographic exposure with 5-μg poly(A)$^+$ RNA samples. MR22 cDNA hybridized to

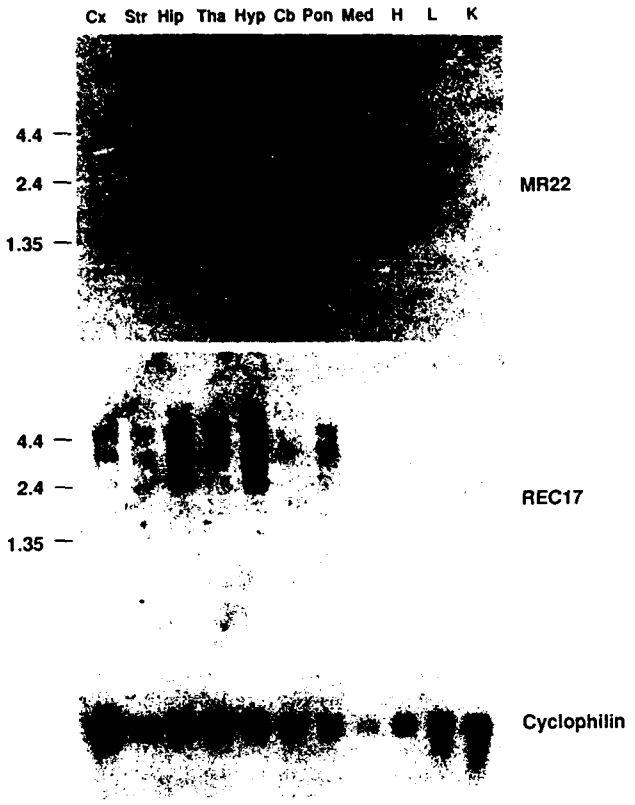

FIG. 3. Northern blot detection of MR22, REC17, and cyclophilin mRNAs. Tissues analyzed: Cx, cortex; Str, striatum; Hip, hippocampus; Tha, thalamus; Hyp, hypothalamus; Cb, cerebellum; Pon, pons; Med, medulla; H, heart; L, liver; K, kidney. Five micrograms of poly(A)$^+$ RNA was used per lane (except medulla, 1.3 μg). Migration of size (kb) markers is shown at left.

three distinct RNAs of 1.5, 1.8, and 3.0 kb that were detectable only in the hippocampal sample (Fig. 3). This was somewhat surprising, given that we originally isolated the MR22 PCR cDNA and the subsequent cDNA clone from a hypothalamus cDNA library. The probable explanation is that MR22 is expressed in the hypothalamus but at a level below detection by Northern blots; this is consistent with our isolation of only one distinct MR22 cDNA clone from a hypothalamic cDNA library containing $>10^6$ recombinants.

In contrast, REC17 detected RNA species of approximately 3.8 and 4.5 kb in most regions of rat brain (Fig. 3), with the highest concentrations in hippocampus and hypothalamus, and lower concentrations in cortex, thalamus, pons, striatum, and medulla. Neither MR22 nor REC17 mRNAs were detectable in heart, kidney, and liver.

We conducted *in situ* hybridization studies using a sensitive method (28, 29) to determine the cellular location of MR22 mRNAs within rat brain (Fig. 4). The most intense hybridization was in the hippocampus, medial and lateral habenular nuclei (Fig. 4 *A*, *C*, and *D*), and raphe nuclei (data not shown). Within the hippocampus, MR22 mRNA was exclusively detected in the CA1 region and subiculum; within CA1, MR22 hybridization was most prominent over the pyramidal layer (Fig. 4 *B* and *E*). Very low silver grain densities, just above background, were found in piriform cortex and supraoptic nucleus of hypothalamus (data not shown). Thus, the cellular localization of MR22 mRNA is consistent with our Northern blot data. Our preliminary *in situ* hybridization data for REC17 are also consistent with our Northern data; REC17 mRNAs were detectable in many regions of the rat central nervous system (piriform cortex, hippocampus, amygdala, septum, and several thalamic nuclei; data not shown). A survey will be presented elsewhere.

The ventral ascending serotonergic pathway originating from the mesencephalic raphe nuclei innervates a number of rostral structures, including the medial habenula and hippocampus but also including the hypothalamus, amygdala, and several cortical regions. The significance of our observation that MR22 mRNAs in the hippocampus are mostly restricted to CA1 is unclear, although the rat 5-HT$_{1B}$ receptor, which is expressed in many areas of the brain, appears to be restricted in the hippocampus to synaptic terminals of CA1 efferents (7). Studies with specific antisera to MR22 and REC17 will contribute to understanding the role these two receptors play in rat central nervous system function.

Classification of MR22 and REC17 Within the G-Protein-Associated 5-HT Receptor Family. Historically, the classification of 5-HT receptors has been based on their pharmacological properties (33). Thus, 5-HT$_1$-like receptors are (*i*) potently antagonized by methiothepin and/or methysergide, (*ii*) not antagonized by molecules binding specifically to 5-HT$_2$, and (*iii*) very sensitive to the agonist 5-carboxamidotryptamine, which shows activity greater than or equal to that of 5-HT (34). With the advent of molecular cloning, 5-HT receptor classification has changed from being based purely

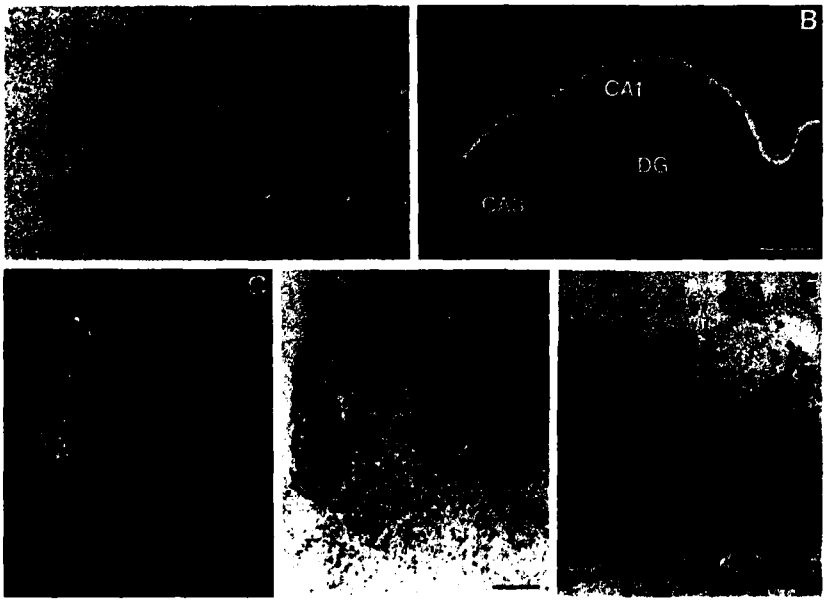

FIG. 4. *In situ* hybridization studies of coronal sections from rat brain of MR22 ^{35}S-labeled antisense RNAs. (*A*) Film autoradiography showing a coronal section hybridized to a MR22 single-stranded ^{35}S-labeled RNA probe. Signal is most prominent in the CA1 region and habenula. Cx, cerebral cortex; Hb, habenula; Hip, hippocampus; Tha, thalamus. (Bar = 2 mm.) (*B*) Dark-field view of the hippocampus hybridized with the MR22 probe. Notice strong hybridization signals in the pyramidal layer of the CA1 area and subiculum. DG, dentate gyrus. (Bar = 500 μm.) (*C*) Dark-field photomicrograph of habenular nuclei. Silver grains are found most densely in the medial habenular nucleus, though some strongly labeled cells are also seen in the lateral nucleus. (*D*) Bright-field photograph of *C*. Mhb, medial habenular nucleus; Lhb, lateral habenular nucleus. (Bar = 200 μm.) (*E*) High-power magnification of a hippocampal section hybridized with the MR22 RNA probe. Notice that signal is seen mostly in pyramidal cells of the CA1 region. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. (Bar = 50 μm.)

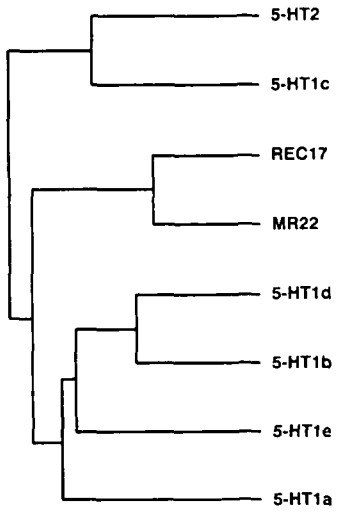

FIG. 5. Dendrogram of the G-protein-associated 5-HT receptor family based on amino acid sequence similarity. Length of each horizontal line is inversely proportional to primary structure identity. Species from which primary structures were derived: 5-$HT_{1A,1B,1C,2}$, rat; 5-HT_{1E}, human; 5-HT_{1D}, dog.

on pharmacological criteria to a more structurally based definition. Hartig (35) and Peroutka (36) have suggested that the receptor nomenclature for 5-HT receptors be primarily based on amino acid sequence. The use of primary structure to catalog 5-HT receptors is appealing because it allows an absolute identification of each 5-HT receptor.

By pharmacological criteria both MR22 and REC17 would probably fall into the 5-HT_1-like class because of their antagonism by methiothepin and agonism by 5-carboxamidotryptamine. We compared their sequences to those of all known 5-HT receptors, using PILEUP (Genetics Computer Group, University of Wisconsin), which ranked (by pairwise sequence identity) each receptor in relation to the others being examined. When the relationships were plotted as a dendrogram (Fig. 5) in which sequence identity is inversely proportional to the length of the horizontal line, REC17 and MR22 were seen to form a distinct subfamily between that of the 5-HT_1-like (5-$HT_{1A/B/D/E}$) and 5-HT_2-like families (5-$HT_{1C/2}$). The primary structure and pharmacological profiles of MR22 and REC17 are consistent with the hypothesis that these receptors belong to a distinct subfamily with 5-HT_1-like class pharmacology. Because the primary structures of REC17 and MR22 are distinct from those of known 5-HT receptors, we propose that REC17 and MR22 be designated as 5-$HT_{5\alpha}$ and 5-$HT_{5\beta}$, respectively. Plassat *et al.* (37) recently identified the apparent mouse homolog of REC17, which they referred to as 5-HT_5.

Concluding Remarks. Previously cloned 5-HT receptors have fallen into either 5-HT_1 or 5-HT_2 families by both significant sequence identity and pharmacological profiles. The two 5-HT receptors reported here have no greater sequence identity to the known 5-HT receptors than to other previously cloned cation-binding receptors. Our data also suggest that these two putative G-protein-associated receptors are not coupled to adenylate cyclase activity. The molecular cloning of all 5-HT receptors will enable pharmacologists to study each receptor in isolation; this in turn will allow the development of rational drug design, therefore leading to successful therapeutic reagents.

We thank Ana Dopazo for providing her rat hypothalamic cDNA library, Dolan Pritchet for his generous contribution of the 5-HT_{1A} receptor gene, Lee Limbird for providing COS-M6 cells and providing advice on transfection experiments, David Julius for the 3T3 fibroblasts expressing rat 5-HT_{1C} and 5-HT_2 receptors, Cheryl Craft for rat hypothalami, and Linda Elder for assistance in preparation of the manuscript. L.d.L. is supported by Commissio Interdepartmental de Recerca i Innovacio Tecnologica. This research was supported in part by National Institutes of Health Grants GM32355 and NS22347 and Air Force Office of Scientific Research Grant F49620-92-J-0188.

1. Steinbusch, H. W. M. (1984) in *Handbook of Chemical Neuroanatomy: Classical Transmitters and Transmitter Receptors in the CNS*, eds. Bjorklund, A., Hokfelt, T. & Kuhar, M. J. (Elsevier, Amsterdam), Part II, Vol. 3, pp. 68–125.
2. Cowen, P. J. (1991) *Br. J. Psychol.* **159,** 7–14.
3. Lucki, I. (1992) *Neurosci. Biobehav. Rev.* **16,** 83–93.
4. Albert, P. R., Zhou, Q.-Y., Va To, H. H. M., Bunzow, J. R. & Civelli, O. (1990) *J. Biol. Chem.* **265,** 5825–5832.
5. Hamblin, M. W., Metcalf, M. A., McGuffin, R. W. & Karpells, S. (1992) *Biochem. Biophys. Res. Commun.* **184,** 752–759.
6. Adham, N., Romanienko, P., Hartig, P., Weinshank, R. L. & Branchek, T. (1992) *Mol. Pharmacol.* **41,** 1–7.
7. Voigt, M. M., Laurie, D. J., Seeburg, P. H. & Bach, A. (1991) *EMBO J.* **10,** 4017–4023.
8. Jin, H., Oksenberg, D., Ashkenazi, A., Peroutka, S. J., Duncan, A. M. V., Rozmahel, R., Yang, Y., Mengod, G., Palacios, J. M. & O'Dowd, B. F. (1992) *J. Biol. Chem.* **267,** 5735–5738.
9. Maroteaux, L., Saudou, F., Amlaiky, N., Boschert, U., Plassat, J. L. & Hen, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3020–3024.
10. Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. (1988) *Science* **241,** 558–564.
11. Lubbert, H., Hoffman, B., Snutch, T. P., van Dyke, T., Levine, A. J., Hartig, P. R., Lester, H. A. & Davidson, N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4332–4336.
12. Hamblin, M. W. & Metcalf, M. A. (1991) *Mol. Pharmacol.* **40,** 143–148.
13. Zgombick, J. M., Weinshank, R. L., Macchi, M., Schechter, L. E., Branchek, T. A. & Hartig, P. R. (1991) *Mol. Pharmacol.* **40,** 1036–1042.
14. Weinshank, R. L., Zgombick, J. M., Macchi, M. J., Branchek, T. A. & Hartig, P. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3630–3634.
15. Levy, F. O., Gudermann, T., Perez-Reyes, E., Birnbaumer, M., Kaumann, A. J. & Birnbaumer, L. (1992) *J. Biol. Chem.* **267,** 7553–7562.
16. McAllister, G., Charlesworth, A., Snodin, C., Beer, M. S., Middlemiss, D. N., Iversen, L. L. & Whiting, P. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5517–5521.
17. Pritchett, D. B., Bach, A. W. J., Wozny, M., Taleb, O., Dal Toso, R., Shih, D. C. & Seeburg, P. H. (1988) *EMBO J.* **7,** 4135–4140.
18. Foguet, M., Hoyer, D., Pardo, L. A., Parekh, A., Kluxen, F. W., Kalkman, H. O., Stuhmer, W. & Lubbert, H. (1992) *EMBO J.* **11,** 3481–3487.
19. Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M. & Julius, D. (1991) *Science* **254,** 432–437.
20. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.-J., Dumont, J. E. & Vassart, G. (1989) *Science* **244,** 569–572.
21. Lovenberg, T. W., Erlander, M. G., Baron, B. M., Racke, M., Slone, A. L., Siegel, B. W., Craft, C., Burns, J. E., Danielson, P. E. & Sutcliffe, J. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2184–2188.
22. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
23. Kobilka, B. K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T. S., Francke, U., Lefkowitz, R. J. & Caron, J. G. (1987) *Nature (London)* **329,** 75–79.
24. Kobilka, B. K., Dixon, R. A. F., Friella, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G. & Lefkowitz, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 46–50.
25. Chirgwin, J. M., Przybyla, A. E., Macdonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
26. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
27. Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. & Sutcliffe, J. G. (1988) *DNA* **7,** 261–267.
28. Gall, C. M. & Isackson, P. M. (1989) *Science* **245,** 758–761.
29. Lecea, L. de, Soriano, E., Del Rio, J. A. & Forss-Petter, S. (1992) *Soc. Neurosci. Abst.* **18,** 1119.
30. Strader, C. D., Sigal, I. S. & Dixon, R. A. (1989) *FASEB J.* **3,** 1825–1832.
31. Middlemiss, D. N. & Tricklebank, M. D. (1992) *Neurosci. Biobehav. Rev.* **16,** 75–82.
32. Cheng, Y. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108.
33. Frazer, A., Maayani, S. & Wolfe, B. B. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 307–348.
34. Bradley, P. B., Engel, G., Feniuk, W., Fozard, J. R., Humphrey, P. P. A., Middlemiss, D. N., Mylecharane, E. J., Richardson, B. P. & Saxena, P. R. (1986) *Neuropharmacology* **25,** 563–576.
35. Hartig, P. R. (1989) *Trends Pharmacol. Sci.* **10,** 64–69.
36. Peroutka, S. J. (1992) *Neuropharmacology* **31,** 609–613.
37. Plassat, J.-L., Boschert, U., Amlaiky, N. & Hen, R. (1992) *EMBO J.* **11,** 4779–4786.

Neuron, Vol. 11, 449–458, September, 1993, Copyright © 1993 by Cell Press

A Novel Adenylyl Cyclase–Activating Serotonin Receptor (5-HT$_7$) Implicated in the Regulation of Mammalian Circadian Rhythms

Timothy W. Lovenberg,* Bruce M. Baron,† Luis de Lecea,* Joseph D. Miller,‡ Rebecca A. Prosser,‡ Michael A. Rea,§ Pamela E. Foye,* Margaret Racke,† Amy L. Slone,† Barry W. Siegel,† Patria E. Danielson,* J. Gregor Sutcliffe,* and Mark G. Erlander*

*Department of Molecular Biology
The Scripps Research Institute
La Jolla, California 92037
†Marion Merrell Dow Research Institute
Cincinnati, Ohio 45215
‡Center for Sleep and Circadian Neurobiology
Stanford University School of Medicine
Stanford, California 94305
§Circadian Neurobiology Research Group
Armstrong Laboratory
Brooks Air Force Base, Texas 78235

Summary

We report the cloning and characterization of a novel serotonin receptor, designated as 5-HT$_7$, which is coupled to the stimulation of adenylyl cyclase. 5-HT$_7$ mRNA is expressed discretely throughout the CNS, predominantly in the thalamus and hypothalamus. 5-HT$_7$ has a unique pharmacological profile that redefines agonist and antagonist classification of ligands previously thought to be "selective." The circadian phase of spontaneous neuronal activity of the rat suprachiasmatic nucleus of the hypothalamus advances in response to serotonin ligands with a pharmacological profile consistent exclusively with that of 5-HT$_7$. These findings suggest a physiological role in the regulation of circadian rhythms for one subtype of serotonin receptor, 5-HT$_7$, and provide a pharmacological test to evaluate its role in other neuronal systems.

Introduction

The circadian organization of behavior has long been studied in an effort to understand how complex organisms anticipate and adapt to the social and light/dark cues they encounter on a daily basis. When circadian rhythmicity is disturbed, humans suffer from mental fatigue and depression (Schwartz, 1993). In mammals, the endogenous clock controlling circadian rhythms is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ralph et al., 1990). Several factors, including neurohumoral agents, light, activity, and diet, influence the endogenous clock (Rusak and Zucker, 1979; Meijer and Rietveld, 1989). One factor, the neurotransmitter serotonin (5-hydroxytryptamine [5-HT]), has been shown in vivo and in vitro to reset or phase shift circadian rhythms of neuronal activity in the SCN (Prosser et al., 1990; Medanic and Gillette, 1992; Shibata et al., 1992; Tominaga et al., 1992; Prosser et al., 1993; Edgar et al., 1993).

Serotonergic pathways emanate primarily from the midbrain raphe nuclei and terminate throughout the brain, including the SCN of the hypothalamus (Jacobs and Azmitia, 1992; Zifa and Fillion, 1992). Physiological actions of 5-HT are transduced through receptors located on both pre- and postsynaptic neuronal membranes. Presently, 11 distinct mammalian genes encoding G protein–coupled 5-HT receptors have been cloned: five receptors (5-HT$_{1A,1B,1D,1E,1F}$) are coupled to the inhibition of adenylyl cyclase (Albert et al, 1990; Voigt et al., 1991; Zgombick et al., 1991; McAllister et al., 1992; Amlaiky et al., 1992; Lovenberg, et al., 1993), three (5-HT$_{1C,2,2F}$) are coupled to phosphatidylinositol-4,5-bisphosphate hydrolysis (Julius et al., 1988; Pritchett et al., 1988; Foguet et al., 1992), two (5-HT$_{5A,5B}$) have unknown coupling mechanisms (Plassat et al., 1992; Erlander et al., 1993), and one (5-HT$_6$) is coupled to the stimulation of adenylyl cyclase (Monsma et al., 1993). In addition, the 5-HT$_3$ receptor gene has been cloned, and this receptor belongs to the family of ligand-gated ion channels (Maricq et al., 1991).

Previous work has shown that the mRNAs encoding the 5-HT$_{1B}$ and 5-HT$_{1C}$ receptors are expressed in the SCN (Roca et al., 1992, Soc. Neurosci., abstract). However, current pharmacological evidence is not consistent with a role for either of these receptor subtypes in 5-HT-induced circadian phase shifts. Therefore, to understand the role of 5-HT in circadian rhythms and hypothalamic functions in general, we initiated research aimed at the discovery of novel G protein–coupled receptors expressed within the hypothalamus. Our initial results, described elsewhere, led to the discovery of 5-HT$_{1F}$, a novel member of the 5-HT$_{1E}$ receptor family (Lovenberg et al., 1993), and 5-HT$_{5A}$ and 5-HT$_{5B}$, members of a new 5-HT receptor family (Erlander et al., 1993). Although mRNAs encoding these receptors have distinct distributions within the rat CNS and unique pharmacological properties, none of their features suggested a role in circadian rhythms. To identify additional members of the recently characterized family of 5-HT$_5$ receptors, we designed a polymerase chain reaction (PCR)–based cloning strategy. We describe here the cloning and functional expression of a gene encoding a putatively G protein–coupled 5-HT receptor (5-HT$_7$) that is linked to the stimulation of adenylyl cyclase. Additionally, we provide evidence that 5-HT$_7$ may mediate 5-HT–induced phase shifts of the circadian rhythm of spontaneous neuronal activity within the SCN.

Results and Discussion

Targeted Cloning Strategy for G Protein–Coupled Receptors

We used a PCR strategy similar to that previously described (Libert et al., 1989; Lovenberg et al., 1993; Erlander et al., 1993) to target and identify a novel G

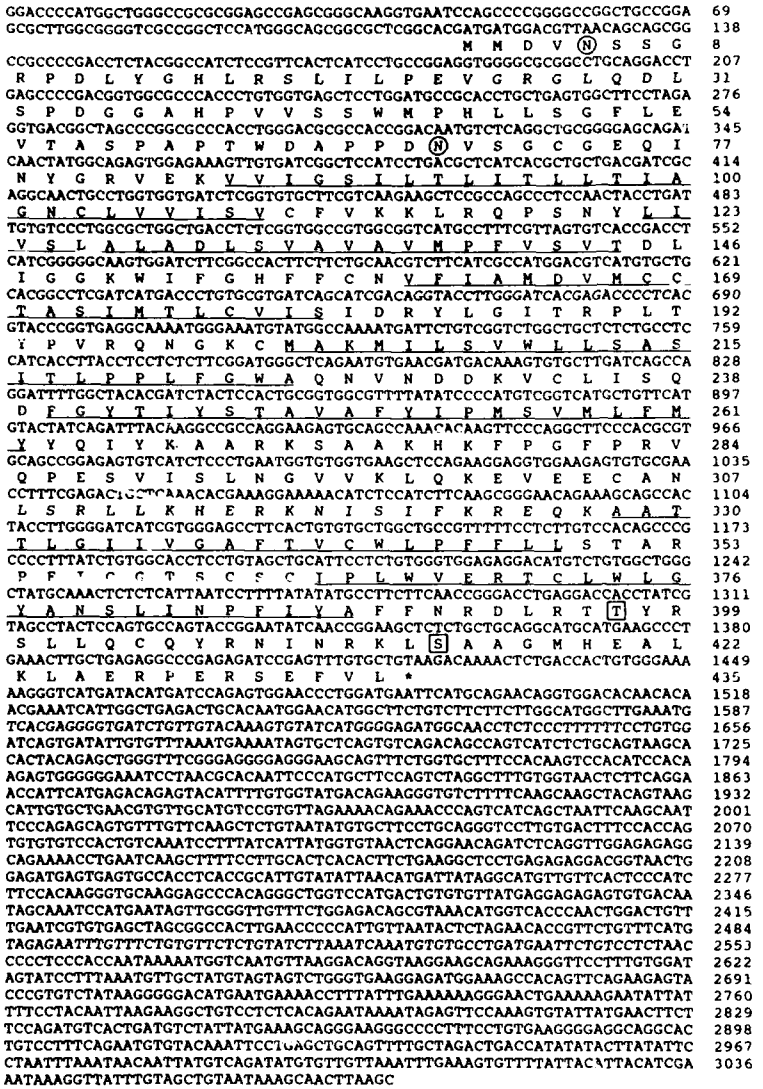

Figure 1. Nucleotide and Predicted Amino Acid Sequence of the Rat REC20 cDNA Clone

The amino acid translation was determined for the longest open reading frame, which begins with a methionine. The region surrounding the codon ATG for translation initiation conforms with the consensus sequence described by Kozak (1984). The putative transmembrane spanning regions are underlined. Potential sites for N-linked glycosylation, protein kinase C phosphorylation, and cAMP-dependent phosphorylation are marked by circles, squares (T), and squares (S), respectively.

protein-coupled receptor. Briefly, degenerate oligonucleotide primers corresponding to the conserved amino acid sequences in putative transmembrane domains III and VII of all known 5-HT receptors were used in a PCR to amplify specific cDNAs from rat brain. The product was used as template for a second PCR using a set of highly degenerate primers corresponding to regions conserved only between the 5-HT_{5A} and 5-HT_{5B} receptor genes, which we recently identified (Erlander et al., 1993). The products of the second reaction were cloned and probed with radiolabeled oligonucleotides specific for 5-HT_{5A} and 5-HT_{5B}. One of the clones that did not hybridize to these probes was picked, and its sequence was determined. The sequence had similarity with known G protein-coupled receptors, and the clone was therefore used to screen a rat hypothalamus cDNA library. We isolated and determined the nucleotide sequence of a 3.6 kb clone (REC20) that exhibited an open reading frame encoding a putative protein of 435 amino acids (Figure 1). Hydrophobicity analysis of the REC20 amino acid sequence predicted the presence of seven major membrane-spanning hydrophobic domains. As shown in Figure 1, the putative receptor encoded by REC20 contains potential sites for N-linked glycosylation and protein kinase C phosphorylation. REC20 also contains the transmembrane-specific amino acid residues (Asp-164, Pro-254, Tyr-262, Phe-339, Trp-343, Pro-345, Trp-374, Asn-383, Pro-384) conserved among all biogenic amine receptors (Trump-Kallmeyer et al., 1992).

Since the 3.6 kb clone contained more than 600 bp of apparent 5′ untranslated sequence, we used an anchored PCR protocol to determine the actual 5′ end of the mRNA. From whole-brain cDNA, we could confirm the existence only of an mRNA that terminated 116 bp upstream from the first initiation codon. We have included only those 116 bp in Figure 1, as it has yet to be confirmed whether the remaining 500 bp of the original clone is part of an actual mRNA. (Never-

theless, Northern blot analyses presented below suggest that longer REC20 RNAs exist). More importantly, the 5′ untranslated 116 bp contains a stop codon in frame with the first initiation codon.

REC20 Is a Novel 5-HT Receptor

The REC20 amino acid sequence was compared with all known G protein–coupled receptors. The greatest similarity was observed within the 5-HT receptor family. Within the conserved transmembrane domains of known 5-HT receptors, REC20 exhibited greatest identity (54%) with the Drosophila 5-HT-dro1 receptor (Witz et al., 1990). However, the entire REC20 coding sequence did not exhibit preferential homology with any known 5-HT receptor subgroup (5-HT-dro1, 39% identity; 5-HT$_{1D}$, 39%; 5-HT$_{1A}$, 37%; 5-HT$_{1E}$, 39%; 5-HT$_5$, 33%) and was therefore provisionally classified as the first member of a novel 5-HT receptor subgroup.

To confirm the hypothesis that REC20 encoded a 5-HT receptor, we performed ^{125}I-labeled lysergic acid diethylamide (LSD) binding with membranes prepared from CosM6 cells transiently expressing REC20 (cloned in pCMV4 mammalian expression vector). LSD is a nonselective 5-HT receptor antagonist that has been shown to bind to all 5-HT receptor subtypes. [^{125}I]LSD bound to REC20 in a saturable manner with high affinity (K_D = 1.2 nM), and its binding was competitively antagonized by 5-HT (IC_{50} = 9 ± 2 nM), but not by melatonin, dopamine, or norepinephrine (Table 1).

REC20 Has High Affinity for Both 5-HT$_{1A}$ Agonists and 5-HT$_{2/1C}$ Antagonists

To characterize the pharmacological profile of REC20 further, we analyzed the binding of a variety of ligands that display affinity for other 5-HT receptors (Table 1). Two notable features of REC20 binding were that the 5-HT$_{1A,1D}$-selective agonist 5-carboxamidotryptamine (5-CT) and the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) were high affinity ligands and that the 5-HT$_{1C/2}$-selective antagonists mesulergine and ritanserin were also high affinity ligands (Table 1). Although 5-HT$_{1A}$-selective agonists exhibited high affinity for REC20, the β-adrenergic antagonist pindolol, which has been shown to exhibit high affinity for 5-HT$_{1A}$ receptors (K_i = 24 nM; Guan et al., 1992), had relatively low affinity for REC20. Thus, pindolol antagonism provides a valuable means to discriminate the effects of 5-HT$_{1A}$ agonists on 5-HT$_{1A}$ versus REC20 receptors. Other nonselective 5-HT receptor ligands, methiothepin, metergoline, and methysergide, also displayed high affinity for REC20. The rank order of affinity of all of the above serotonergic ligands for REC20 does not correlate with that of any of the previously described 5-HT receptors, including that for 5-HT$_{1A}$ (Table 1) or for the putative 5-HT$_4$ receptor (Bockaert et al., 1992). The experimentally determined IC_{50} values for 5-HT ligands versus the 5-HT$_{1A}$ receptor were included for a comparison with REC20. Some of these values are different from several reported literature values. We believe that this is a reflection of the use of [^{125}I]LSD to label the receptors. As a point of interest, REC20 exhibits high affinity for the atypical antipsychotic agent clozapine (<50 nM; data not shown). It remains to be determined whether REC20 is a physiological substrate for in vivo actions of clozapine.

REC20 Is Positively Coupled to Adenylyl Cyclase

To determine the signal transduction pathway of REC20 activation, HeLa cells were transfected with the receptor gene, and cyclic AMP (cAMP) accumulation in response to 5-HT agonists was measured as previously described (Baron and Siegel, 1989). cAMP accumulation in REC20-transfected cells increased in a dose-dependent manner in response to 5-HT, 5-CT, and 8-OH-DPAT (Figure 2A). The rank order of potency (5-CT > 5-HT > 8-OH-DPAT) for these agonists was the same as that for receptor binding, with EC_{50} values of 13 ± 2 nM, 156 ± 37 nM, and 2345 ± 940 nM, respectively (n = 3). The observation that these values are all about 15- to 20-fold higher than the IC_{50} values for competition with [^{125}I]LSD binding may reflect low affinity binding states, since agonists exhibit different binding profiles in whole-cell versus membrane preparations. Additionally, as discussed below, antagonist K_i values are identical between binding and functional assays.

Stimulation of cAMP accumulation by 5-CT was competitively blocked by the 5-HT$_{2/1C}$ antagonist mesulergine, the 5-HT$_{1C/2}$ antagonist ritanserin, and the 5-HT$_{1A}$ antagonist NAN-190 (Glennon et al., 1988) (Figure 2B), but not by the 5-HT$_{1A}$ antagonist pindolol at 10 μM (data not shown). To characterize the kinetic

Table 1. [^{125}I]LSD Binding to Rat REC20 Receptors

Ligand	REC20 IC_{50} (nM ± SE)	5-HT$_{1A}$ IC_{50} (nM ± SE)
5-CT	0.83 ± .03	21 ± 3
Methiothepin	1.30 ± .01	16 ± 5
5-HT	9 ± 2	245 ± 77
Mesulergine	17 ± 3	>1000
Metergoline	30 ± 12	500 ± 46
Methysergide	32 ± 1	>1000
Ergotamine	32 ± 7	5 ± 1
Ritanserin	60 ± 21	>1000
8-OH-DPAT	98 ± 7	49 ± 5
NAN-190	145 ± 10	0.6[a]
Buspirone	687 ± 44	126 ± 14
Zacopride	>1000	–
Sumatriptan	>1000	–
Pindolol	>1000	57
Norepinephrine	>1000	>1000
Dopamine	>1000	>1000

Binding was analyzed as described by Baron and Siegel (1990). CosM6 cells were transfected with expression vectors (pCMV4) containing either REC20 or 5-HT$_{1A}$ (Kobilka et al., 1987) cDNAs. Cell membranes were labeled with [^{125}I]LSD in the presence of various concentrations of unlabeled ligands. IC_{50} values represent the concentrations of ligands (nM ± SE) at which binding of [^{125}I]LSD could be reduced by 50%.

[a] Glennon et al., 1988.

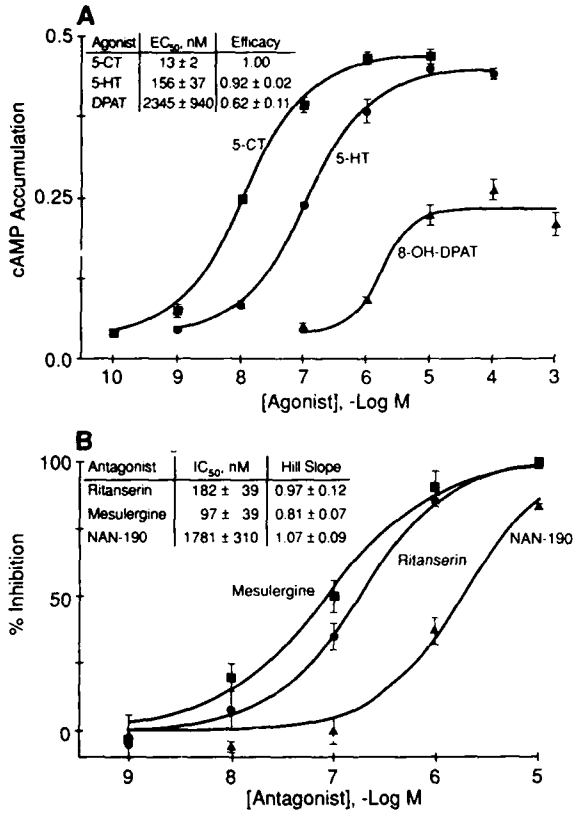

Figure 2. Agonists Stimulate cAMP Accumulation in HeLa Cells Transfected with REC20

(A) cAMP accumulation in response to 5-CT, 5-HT, and 8-OH-DPAT are marked by squares, circles, and triangles, respectively. cAMP accumulation is measured as the percent conversion of [^{3}H]ATP to [^{3}H]cAMP.

(B) cAMP accumulation was measured in response to 100 nM 5-CT in the presence of several 5-HT antagonists and is recorded as percent inhibition. Mesulergine, ritanserin, and NAN-190 are marked by squares, circles, and triangles, respectively.

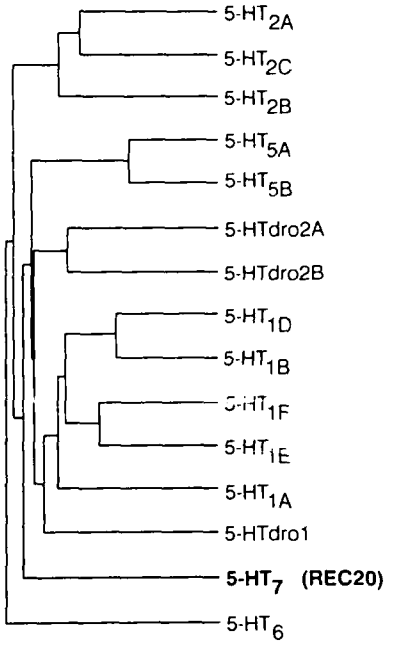

Figure 3. Dendrogram Analysis of 5-HT G Protein-Coupled Receptor Family Demonstrating How Subgroups of 5-HT Receptors Cluster According to Amino Acid Sequence Similarity

The lengths of the horizontal bars are inversely proportional to the similarity between sequences. The Drosophila 5-HT-dro1, 5-HT-dro2A, and 5-HT-dro2B (Witz et al., 1990; Saudou et al., 1992) receptors were included to show sequence relationships. Amino acid sequence data were compiled and analyzed using the Genetics Computer Group (Madison, WI) sequence software Lineup and Pileup (Devereux et al., 1984).

mechanism of the blockade of adenylyl cyclase activity, we conducted detailed concentration-response curves for 5-CT in the presence of 0, 100, 300, and 1500 nM mesulergine. In the presence of the antagonist, there was a concentration-dependent rightward shift in the EC_{50} for 5-CT (values were 13, 196, 700, and 3754 nM, respectively), unaccompanied by changes in either maximal responses or the Hill slope. Transformation of the data by the method of Arunlakshana and Schild (1949) yielded a line of slope 1.09 and x-intercept of 8.79. These data are consistent with mesulergine being a competitive inhibitor with apparent K_D of 8.5 nM. This value is nearly identical to the K_i value obtained for mesulergine in the binding assays (8.8 nM). This confirms the competitive and specific nature of these antagonist interactions.

Overall, these findings raise issues related to previous discriminations of 5-HT function through the use of available "selective" ligands, which must now be reinterpreted with respect to REC20, a receptor that operates by an effector-coupling mechanism distinct from that of 5-HT_{1A} and 5-$HT_{1C/2}$ receptors. We have not tested whether REC20 couples to other second messenger systems such as phospholipase C or phospholipase A_2. It may be interesting to test this in other cell types.

REC20 by Current Nomenclature Is the 5-HT_7 Receptor

The current classification proposed for 5-HT receptors subdivides them into subgroups based on both functional and structural criteria (Humphrey et al., 1993). 5-HT_1 receptors share structural similarity and are all linked to the inhibition of adenylyl cyclase. The 5-HT_2 receptors stimulate phosphoinositide metabolism. The 5-HT_{1C} receptor shares considerable sequence and functional similarity with this group (5-HT_{2A} [formerly 5-HT_2] and 5-HT_{2B} [formerly 5-HT_{2F}]) and is now proposed to be classified as the 5-HT_{2C} receptor (Humphrey et al., 1993). The 5-HT_3 receptor is a ligand-gated ion channel with no structural similarity to G protein-coupled receptors. The putative 5-HT_4 receptor, which has been proposed to stimulate adenylyl cyclase, has only been identified pharmacologically and is yet to be cloned. The recently identified 5-HT_5 receptors have unique structural properties and, as of yet, have not been functionally linked to any second messenger signaling systems. Recently, a novel serotonin recep-

tor (5-HT$_6$) that is positively coupled to adenylyl cyclase was described. The 5-HT$_6$ designation has previously been applied to a novel mouse receptor, which has been subsequently identified as the 5-HT$_{1E\beta}$ (5-HT$_{1F}$) receptor (Amlaiky et al., 1992; Adham et al., 1993). Although REC20 also stimulates adenylyl cyclase, since it is structurally unique, we have classified it provisionally as a new subtype, 5-HT$_7$.

The primary sequence relationship of the entire 5-HT receptor family is graphically illustrated as a dendrogram (Figure 3) that includes the three cloned Drosophila 5-HT receptors, 5-HT-dro1, 5-HT-dro2A, and 5-HT-dro2B. A dendrogram represents a pairwise comparison of amino acid sequences that matches sequences on the basis of similarity with each other compared with the group. It is not the equivalent of a phylogenetic tree. It is clear from this analysis that REC20 represents a separate class of 5-HT receptors. The 5-HT$_7$ designation for REC20 was chosen over 5-HT$_4$ because the pharmacological properties and anatomical distribution (see below) of REC20 vary greatly from those of the putative 5-HT$_4$. We also chose not to classify REC20 as a second 5-HT$_6$ receptor because there is little structural or sequence similarity to make it an immediate family member. Ultimately, the research community will dictate whether the 5-HT receptor nomenclature will base the numerical designation solely on sequence relationships or on a functional (second messenger coupling) grouping. Thus, all adenylyl cyclase–activating receptors may eventually fall into the general class of 5-HT$_4$ receptors. However, to avoid misunderstandings about the identity of REC20, we consider that its uniqueness presently merits separate classification as 5-HT$_7$.

5-HT$_7$ mRNAs Are Predominantly Expressed in the Thalamus and Hypothalamus

We determined by Northern blot analysis the anatomical distribution of 5-HT$_7$ mRNA expression in rat brain and in peripheral tissues. The 5-HT$_7$ clone hybridized to at least two mRNAs between 3 kb and 4.2 kb in length, predominantly in the hypothalamus and thalamus, with significantly lower concentrations in hippocampus, cortex, and medulla (Figure 4). No detectable 5-HT$_7$ mRNAs were found in cerebellum, striatum, heart, liver, or kidney (Figure 4), or in adrenal glands, testes, ovaries, or spleen (data not shown).

To define the anatomical distribution further, we used in situ hybridization in brain sections from adult rats. 5-HT$_7$ expression was prominent in the thalamus, particularly in the anteroventral and paraventricular thalamic nuclei (Figures 5B, 5D, and 5E). Hybridization was also detected in the hippocampus, predominantly in the pyramidal cell layer of the CA2 and CA3 fields (Figures 5D and 5F). In addition, 5-HT$_7$ mRNA was detectable in the piriform and retrosplenial cortices, as well as in layers II and III of the neocortex (Figures 5A–5D). Moderate expression was found in the anterior hypothalamic area, arcuate nucleus (Figure 5G), and other hypothalamic nuclei. We have been unable to establish definitively whether 5-HT$_7$ mRNA is expressed in the SCN. Expression of 5-HT$_7$ mRNA was detected by PCR amplification from histochemically identified SCN tissue punches (data not shown). However, we have not consistently detected 5-HT$_7$ in SCN by in situ hybridization, perhaps because expression is near the threshold of detection by this method. The issue of 5-HT$_7$ expression in the SCN will be resolved when antisera specific for the 5-HT$_7$ receptor protein become available.

5-HT$_7$ Is a Candidate for Mediating 5-HT-Induced Phase Shifts of Neuronal Activity in the SCN

A number of the aspects of 5-HT$_7$ pharmacology, as well as the finding that 5-HT$_7$ mRNA is expressed in the hypothalamus, particularly in neurons surrounding and possibly within the SCN, led us to hypothesize that 5-HT$_7$ may mediate the observed serotonergic regulation of circadian rhythms. Previous investigations have demonstrated that 5-HT agonists induce phase shifts in behavioral circadian rhythms in rats (Edgar et al., 1993) and spontaneous activity of rat SCN neurons in vitro (Prosser et al., 1990). Both in vivo and in vitro studies support the hypothesis that the 5-HT$_{1A}$ or a 5-HT$_{1A}$-like receptor mediates the action of 5-HT in the SCN (Medanic and Gillette, 1992; Shibata et al., 1992; Tominaga et al., 1992; Prosser et al., 1993).

Using the in vitro rat hypothalamic slice preparation, Prosser et al. (1990) initially demonstrated that a 5-HT agonist, quipazine, induced phase advances in neuronal activity during subjective day. Medanic and Gillette (1992) subsequently demonstrated that application of 5-CT, 8-OH-DPAT, or 5-HT induced a phase advance in neuronal activity during subjective day; the optimal time of administration for the sero-

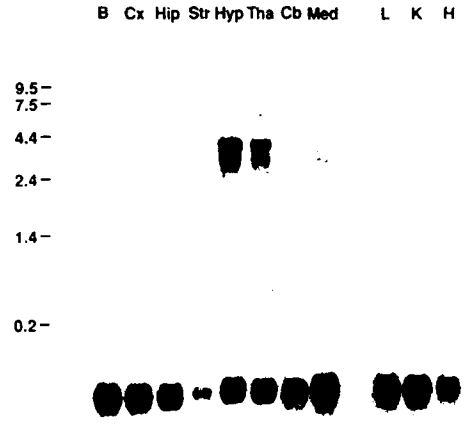

Figure 4. Northern Blot Detection of 5-HT- and Cyclophilin mRNAs

Tissues analyzed: B, brain; Cx, cortex; Hip, hippocampus; Str, striatum; Hyp, hypothalamus; Tha, thalamus; Cb, cerebellum; Med, medulla; L, liver; K, kidney; H, heart. Five micrograms of poly(A)$^+$ RNA was used per lane. Migration of size (kb) markers is shown at left.

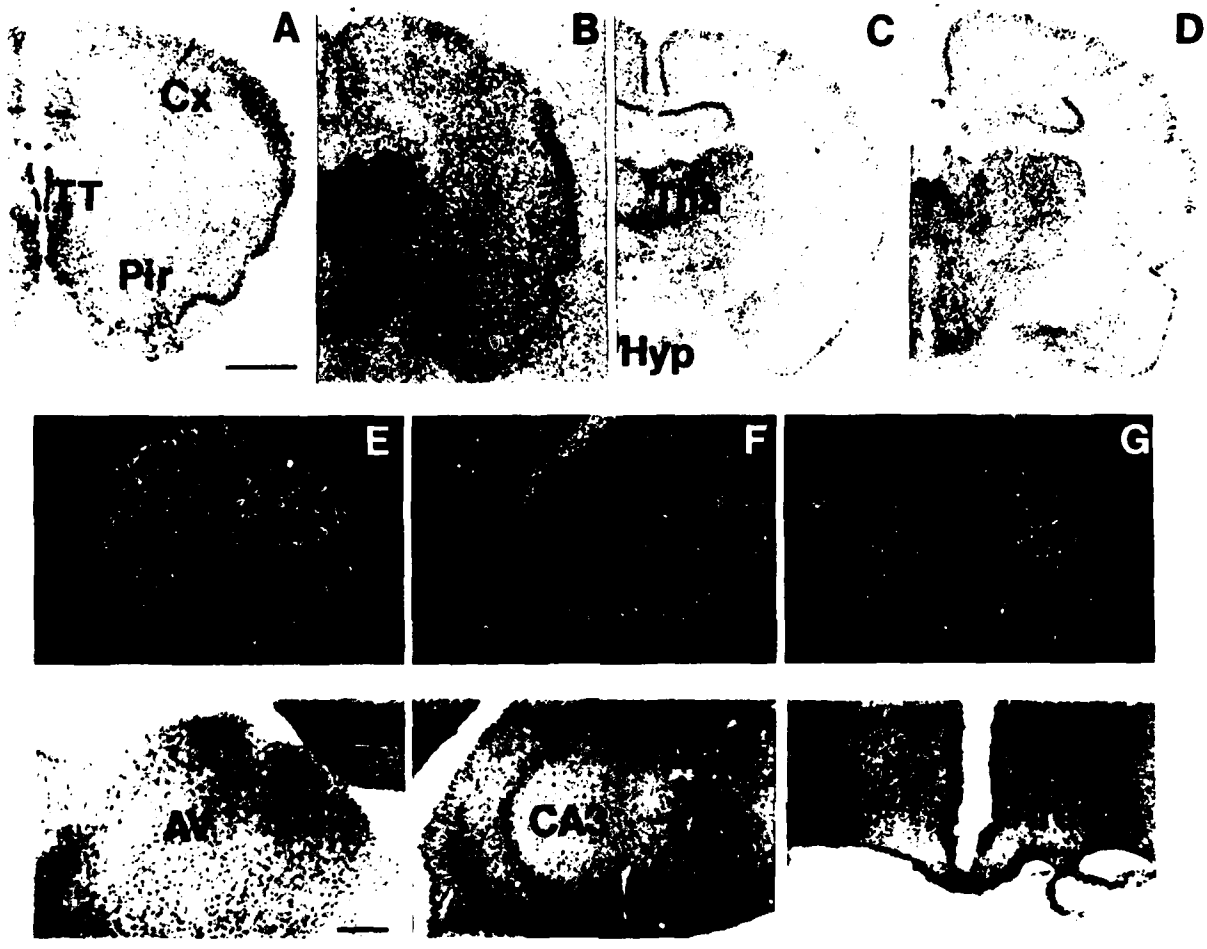

Figure 5. In Situ Hybridization of Rat Brain Coronal Sections with a REC20 Riboprobe

Autoradiograms show intense hybridization in the anteroventral thalamic nucleus (B, E, and E'), paraventricular thalamic nucleus (D), and CA3 field of the hippocampus (D, F, and F'). Moderate signals were found in the outer layers of the cerebral cortex (A–D). Prominent hybridization within the hypothalamus was observed in the arcuate nucleus (G and G'). Arc, arcuate nucleus; AV, anteroventral thalamic nucleus; TT, tenia tecta; Pir, piriform cortex; Cx, cerebral cortex; Tha, thalamus; Hyp, hypothalamus. Bars, 2 mm (A–D); 150 μm (E–G).

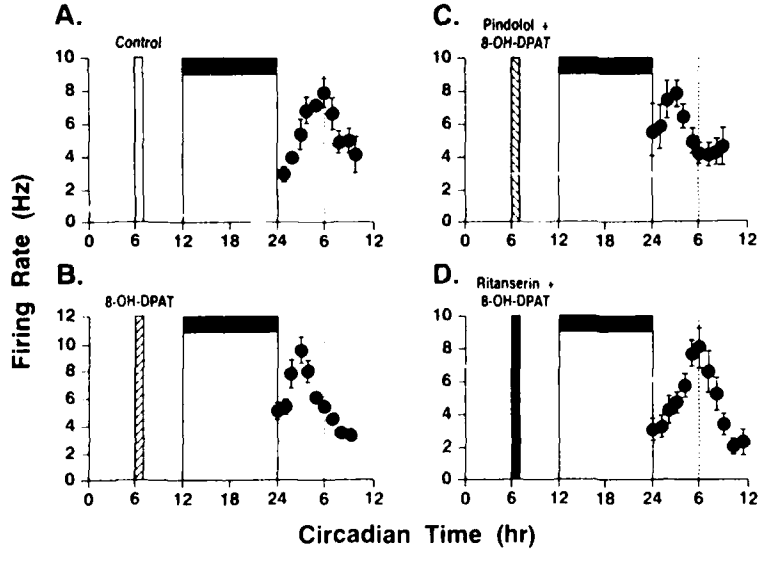

Figure 6. Effects of Serotonergic Agents on SCN Neuronal Activity In Vitro

Plotted are the 2 hr means ± SEM of neuronal activity recorded in single experiments from SCN after vehicle treatment (A), treatment with 5 μM 8-OH-DPAT (B), cotreatment with 1 μM pindolol and 5 μM 8-OH-DPAT (C), and cotreatment of 10 μM ritanserin and 5 μM 8-OH-DPAT (D). Horizontal bars: time of lights off in donor colony (subjective night); vertical bars: time of treatment; dotted line: mean time of peak in untreated slices; closed circles: firing rate.

tonergic agents was CT7 (7 hr after lights on in a 12 hr light: 12 hr dark cycle). Prosser et al. (1993) demonstrated that the 5-HT phase advances are not generated by the 5-HT_{1B} agonist CGS12066B or 5-$HT_{1C/2}$ agonist 1-(2,5-dimethoxy-4-bromophenyl)-2-amino propane (DOB), or by the 5-$HT_{3/4}$ antagonist 3-tropanyl-indole-3-carboxylate (ICS205-930). Such advances are antagonized by the nonspecific antagonist metergoline and the specific 5-HT_{1A} antagonist NAN-190. This pharmacological profile was previously consistent only with 5-HT_{1A} receptor involvement. However, our current studies indicate that this profile is more consistent with 5-HT_7 activation.

To discriminate between the alternative hypotheses of 5-HT_{1A} versus 5-HT_7 receptor involvement, we performed pharmacological analysis designed to differentiate between 5-HT_{1A} and 5-HT_7 receptors. Using the in vitro hypothalamic slice preparation (for review see Gillette, 1991), we found that, as previously described (Prosser et al., 1990), 8-OH-DPAT applied at CT7 caused a 3 hr phase advance in spontaneous neuronal activity (Figure 6B), compared with a control preparation receiving no drug (Figure 6A). Pindolol, which has high affinity for 5-HT_{1A} receptors (K_i = 24 nM; Guan et al., 1992) but not for 5-HT_7 receptors (K_i > 1000 nM; Table 1) at 1 μM, a concentration that should completely saturate 5-HT_{1A} receptors, had no effect on the phase shift (Figure 6C). Ritanserin, an antagonist with high affinity for 5-HT_7 (K_i = 60 nM; Table 1) but not 5-HT_{1A} (>1000 nM), completely antagonized the action of 8-OH-DPAT (Figure 6D). Application of ritanserin alone had no effect (data not shown). Although ritanserin is also a high affinity 5-$HT_{1C/2}$ antagonist, 8-OH-DPAT has very little affinity for 5-$HT_{1C/2}$ receptors (Pazos et al., 1985). Furthermore, DOB, a potent 5-$HT_{1C/2}$ agonist, is completely ineffective in this paradigm (Prosser et al., 1993). These antagonist studies strongly suggest that 5-HT_{1A} cannot be the receptor that mediates the observed 5-HT-induced phase advance. However, the 5-HT_7 receptor remains a candidate.

Further evidence in support of the 5-HT_7 receptor comes from signal transduction studies of Prosser and Gillette (1989), who demonstrated that application of cAMP analogs or phosphodiesterase inhibitors can mimic the effect of 5-HT. Importantly, the effectual peak of cAMP sensitivity during subjective day matches that of 5-HT. Furthermore, Prosser et al. (1992, Soc. Neurosci., abstract) have demonstrated that the competitive inhibitor of cAMP (RpcAMP) and the protein kinase A inhibitor (H8) block quipazine-induced phase advances during subjective day. The above studies, in combination, suggest that the signal transduction pathway induced by 5-HT proceeds via stimulation of adenylyl cyclase, followed by an increase in intracellular cAMP and a subsequent activation of protein kinase A. This signal transduction pathway is consistent with the effector-coupling mechanism of 5-HT_7. In contrast, both rat 5-HT_{1A} expressed in GH_4C_1 pituitary cells (Albert et al., 1990) and human 5-HT_{1A} expressed in HeLa or COS-7 cells (Fargin et al., 1989) mediate inhibition of intracellular cAMP accumulation.

Conclusion

We have identified a novel serotonin receptor, 5-HT_7, that is coupled positively to cAMP. A variety of 5-HT ligands, notably 5-HT_{1A} agonists and 5-$HT_{1C/2}$ antagonists, exhibit high affinity for 5-HT_7. The mRNA encoding the 5-HT_7 receptor is detected only in the brain with the highest levels of expression in the thalamus and hypothalamus. The unique pharmacological profile and the positive coupling to cAMP together make 5-HT_7 a candidate for mediating serotonin-induced circadian phase shifts during the subjective day. It remains to be determined whether 5-HT_7 is in the SCN itself or only in surrounding tissue and how the mechanism of phase shifting works. The 5-HT_7 receptor may prove to be a valuable target for selective compounds for the treatment of jet lag or sleep disorders of circadian nature.

Another important aspect of this work is the demonstration that previously selective 5-HT ligands (e.g., 5-CT, 8-OH-DPAT, and ritanserin) have been shown to have additional pharmacological properties. The 5-HT_7 receptor must now be taken into consideration with respect to the interpretation of experiments that have used these ligands. The identification of new receptor subtypes will greatly enhance our current understanding of the physiology and pharmacology of these receptors and should ultimately lead to improved, rational drug design and therapy.

Experimental Procedures

Cloning Strategy and Sequence Analysis

Oligonucleotides of degenerate sequence were synthesized corresponding to conserved transmembrane domains III and VII of known 5-HT receptors (AIAA/SLDRY and WL/IGYL/SNSL/F, respectively, with the TM VII oligonucleotide as the reverse complement). These oligonucleotides were used to amplify a rat brain cDNA template (pool of whole brain, hypothalamus, hippocampus, and cerebral cortex). The products of this reaction were then used as template in a second reaction with the same TM VII oligonucleotide, but with the 5′ oligonucleotide corresponding to a region (FVYWKIYK) downstream of TM V, conserved only between the 5-HT_{5A} and 5-HT_{5B} receptors. The TM V and TM VII oligonucleotides contained restriction endonuclease sites at their 5′ ends (SalI–KpnI and XbaI, respectively). The products of the second reaction were subcloned into pBluescript (Stratagene, La Jolla, CA) and used to transform DH5α cells (BRL, Bethesda, Md). Lifts from approximately 1000 colonies were probed with a mixture of radiolabeled 20-mer oligonucleotides specific to 5-HT_{5A} and 5-HT_{5B} receptors. Two colonies did not hybridize and were picked and grown, and the sequence of the plasmid inserts was determined. One insert, REC20, was radiolabeled and used to screen a rat hypothalamus cDNA library. Positive clones were purified, digested with EcoRI,and subcloned into pBluescript, and their sequence was determined according to the dideoxy termination method (Sanger et al., 1977) using specific oligonucleotide primers.

Anchored PCR

Poly(A)-selected RNA extracted from rat brain was converted to

first strand cDNA using random hexamer oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA), and the cDNAs were subsequently tailed with dGTP using terminal transferase. Anchored PCR (Frohman et al., 1988) was performed using this dG-tailed cDNA as template. A primer complementary to nucleotides 148–167 (5'AACGGAGATGGCCGTAGAGG3') of known REC20 (Figure 1) sequence and an anchored primer (5'-GCACCGCGGAGCTCAAGCTT(C_{20})-3') were used in an anchored PCR. Ten percent of the product was separated by gel electrophoresis and transferred onto nitrocellulose, and the filter was hybridized with a radioactively labeled oligonucleotide representing nucleotides 110–129 of the REC20 sequence. A major band of approximately 180 bp was detected. The remaining product was digested with HindIII and separated by electrophoresis, and the 180 bp fragment was excised and subcloned into the HindIII site of pBluescript(KS+) for nucleotide sequence determination.

Expression in CosM6 Cells

CosM6 cells (a subclonal line of COS-7 cells, obtained from Edith Womack in the laboratory of J. L. Goldstein, University of Texas Health Sciences, Dallas, and generously provided by L. E. Limbird, Vanderbilt University) or HeLa cells were transfected with pDP5HT1a (Kobilka et al., 1987) or pCMV4REC20 (REC20 cDNA) as described (Lovenberg et al., 1993). [^{125}I]LSD binding was performed exactly as reported (Lovenberg et al., 1993), as adapted from Baron and Siegel (1990). NAN-190, 8-OH-DPAT, zacopride, and pindolol were purchased from Research Biochemicals Inc. (Natick, MA); 5-HT, norepinephrine, and dopamine were purchased from Sigma (St. Louis, MO); clozapine, mesulergine, and methysergide were generous gifts from Sandoz Pharmaceuticals; Sumatriptan, ergotamine, and metergoline were generously provided by Glaxo, Janssen, and Chemodyne, respectively. 5-CT was prepared by Dr. R. Bernotas (Marion Merrell Dow Research Institute, Cincinnati, OH). cAMP accumulation experiments were performed exactly as described in Lovenberg et al. (1993), as adapted from Baron and Siegel (1989).

Schild regression analysis was performed as described by Arunlakshana and Schild (1949). K_i values for ligand-binding antagonism were determined by the method of Cheng and Prusoff (1973), in which $K_i = IC_{50}/(1 + [C]/K_D)$, with [C] as the [^{125}I]LSD concentration.

RNA Blots

Total RNA was isolated from frozen tissues of adult Sprague-Dawley rats by extraction with guanidinium isothiocyanate (Chirgwin et al., 1979). Oligo(dT)-cellulose chromatography (Aviv and Leder, 1972) was used to enrich for poly(A)$^+$ RNA. For RNA blots, 5 μg of poly(A)$^+$ RNA were loaded per lane and subsequently resolved by electrophoresis on a 1.2% agarose, 1.2 M formaldehyde gel, transferred to nitrocellulose membrane, and hybridized to a full-length ^{32}P-labeled REC20 cDNA. To confirm that similar amounts of intact RNA were loaded in each gel lane, blots were stripped and hybridized with a ^{32}P-labeled cDNA probe for ubiquitously expressed cyclophilin mRNA (Danielson et al., 1988).

In Situ Hybridization

Free-floating in situ hybridization was performed as adapted from Gall and Isackson (1989). Coronal sections, 25 μm thick, from four young adult Sprague-Dawley rats (sacrificed during daytime) were hybridized at 55°C for 16 hr with ^{35}S-labeled single-stranded RNA probes at 1.5×10^7 cpm/ml. Free-floating sections were then digested with RNAase A at 4 μg/ml in 50 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA (pH 7.5) for 1 hr at 37°C. Washes were performed in 50% formamide, 75 mM NaCl, 7.5 mM sodium citrate (pH 7), 14 mM 2-mercaptoethanol at 60°C for 3 hr. Further washes were carried out in 15 mM NaCl, 1.5 mM sodium citrate (pH 7), 0.5% sodium N-laurylsarcosine at 68°C for 1 hr. Sections were mounted on coated slides, dehydrated, and exposed to Kodak XAR film for 5 days at room temperature. Autoradiography was performed by dipping slides in Ilford K5 emulsion diluted 1:1 with water and exposing them with desiccant at 4°C for 5 weeks. Slides were developed in Kodak D19, counterstained, and mounted in Permount.

Spontaneous Neuronal Activity of SCN Slices

The procedures used in slice preparation and maintenance, electrophysiological recordings, and data analysis are published elsewhere (Green and Gillette, 1982; Prosser et al., 1993). Slice treatment consisted of stopping perfusion and replacing bath medium for 1 hr with medium containing no additional compounds (control) or 8-OH-DPAT. In blocking experiments, the bath medium was first replaced for 15 min with perfusion medium containing either pindolol or ritanserin (dissolved in 100% ethanol and then diluted to the proper concentration), followed by replacement for 1 hr with medium containing 8-OH-DPAT and either pindolol or ritanserin, followed by an additional 15 min treatment with medium containing pindolol or ritanserin. This was followed by the normal perfusion medium.

Acknowledgments

We thank Ana Dopazo for providing the rat hypothalamus library, David Russell for providing the pCMV4 expression vector, Dolan Pritchett for his generous contribution of the 5-HT_{1A} receptor gene, and Lee Limbird for providing CosM6 cells and for advice on transfection experiments. We also thank Jeffrey E. Burns and Marianna P. Cagle for their excellent technical assistance. We gratefully acknowledge the assistance of Linda Elder in the preparation of this manuscript. This work was supported in part by NIH RO1 NS22347, AFOSR F49620-92-J-0188, AFOSR 2312W6 (M. A. R), and a grant from the Upjohn Company (J. D. M.). L. d. L. was supported by Subdireccio General de Recerca (Generalitat de Catalunya). R. A. P. was supported by NIH postdoctoral fellowship NS08905.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received June 9, 1993; revised July 19, 1993.

References

Adham, N., Kao, H.-T., Schecter, L. E., Bard, J., Olsen, M., Urquhart, D., Durkin, M., Hartig, P. R., Weinshank, R. L., and Branchek, T. A. (1993). Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. Proc. Natl. Acad. Sci. USA *90*, 408–412.

Albert, P. R., Zhou, Q.-Y., Van Tol, H. H. M., Bunzow, J. R., and Civelli, O. (1990). Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine$_{1A}$ receptor gene. J. Biol. Chem. *265*, 5825–5832.

Amlaiky, N., Ramboz, S., Boschert, U., Plassat, J.-L., and Hen, R. (1992). Isolation of a mouse "5HT1E-like" serotonin receptor expressed predominantly in hippocampus. J. Biol. Chem. *267*, 19761–19764.

Arunlakshana, O., and Schild, H. O. (1949). Some quantitative used of drug antagonists. Br. J. Pharmacol. *14*, 48–58.

Aviv, H., and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA *69*, 1408–1412.

Baron, B. M., and Siegel, B. W. (1989). α_2-adrenergic and muscarinic cholinergic receptors have opposing actions on cyclic AMP levels in SK-N-SH human neuroblastoma cells. J. Neurochem. *53*, 602–609.

Baron, B. M., and Siegel, B. W. (1990). p-[^{125}I]iodoclonidine, a novel radiolabeled agonist for studying central α_2-adrenergic receptors. Mol. Pharmacol. *38*, 348–356.

Bockaert, J., Fozard, J. R., Dumuis, A., and Clarke, D. E. (1992). The 5-HT receptor: a place in the sun. Trends Pharmacol. Sci. *13*, 141–145.

Cheng, Y.-C., and Prusoff, W. H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. *22*, 3099–3108.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294–5299.

Danielson, P., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. j., and Sutcliffe, J. G. (1988). p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA *7*, 261–267.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. *12*, 387–395.

Edgar, D. M., Miller, J. D., Prosser, R. A., Dean, R. R., and Dement, W. C. (1993). Serotonin and the mammalian circadian system. II. Phase-shifting rat behavioral rhythms with serotonergic agonists. J. Biol. Rhythms *8*, 17–31.

Erlander, M. G., Lovenberg, T. W., Baron, B. M., deLecea, L., Danielson, P. E., Racke, M., Slone, A. L., Siegel, B. W., Foye, P. E., Cannon, K., Burns, J. E., and Sutcliffe, J. G., (1993). Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. Proc. Natl. Acad. Sci. USA *90*, 3452–3456.

Fargin, A., Raymond, J. R., Reagan, J. W., Cotecchia, S., Lefkowitz, R. J., and Caron, M. G. (1989). Effector coupling mechanisms of the cloned 5-HT$_{1A}$ receptor. J. Biol. Chem. *264*, 14848–14852.

Foguet, M., Hoyer, D., Pardo, L. A., Parekh, A., Fluxen, F. W., Kalkman, H. O., Stuhmer, W., and Lubbert, H. (1992). Cloning and functional characterization of the rat stomach fundus serotonin receptor. EMBO J. *11*, 3481–3487.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA *85*, 8998–9002.

Gall, C. M., and Isackson, P. M. (1989). Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. Science *245*, 758–761.

Gillette, M. U. (1991). SCN electrophysiology in vitro: rhythmic activity and endogenous clock properties. In The Suprachiasmatic Nucleus: The Mind's Clock. D. C. Klein, R. Y. Moore, and S. M. Reppert, eds. (New York: Oxford University Press, Inc.), pp. 125–143.

Glennon, R. A., Naiman, N. A., Pierson, M. E., Titeler, M., Lyon, R. A., and Weisberg, E. (1988). NAN-190: an arylpiperazine analog that antagonizes the stimulus effects of the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT). Eur. J. Pharmacol. *154*, 339.

Green, D. J., and Gillette, R. (1982). Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic slice. Brain Res. *245*, 198–200.

Guan, X.-M., Peroutka, S. J., and Kobilka, B. K. (1992). Identification of a single amino acid residue responsible for the binding of a class of β-adrenergic receptor antagonists to 5-hydroxytryptamine$_{1A}$ receptors. Mol. Pharmacol. *41*, 695–698.

Humphrey, P. P. A., Hartig, P., and Hoyer, D. (1993). A proposed new nomenclature for 5-HT receptors. Trends Pharmacol. Sci., in press.

Jacobs, B. L., and Azmitia, E. C. (1992). Structure and function of the brain serotonin system. Physiol. Rev. *72*, 165–229.

Julius, D., MacDermott, A. B., Axel, R., and Jessell, T. M. (1988). Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science *241*, 558–564.

Kobilka, B. K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T. S., Francke, U., Lefkowitz, R. J., and Caron, M. G. (1987). An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. Nature *329*, 75–79.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucl. Acids Res. *12*, 857–872.

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., VanSande, J., Maenhaut, C., Simons, M.-S., Dumont, J. E., and Vassart, G. (1989). Selective amplification and cloning of four new members of the G protein-coupled receptor family. Science *244*, 569–572.

Lovenberg, T. W., Erlander, M. G., Baron, B. M., Racke, M., Slone, A. L., Siegel, B. W., Craft, C. M., Burns, J. E., Danielson, P. E., and Sutcliffe, J. G. (1993). Molecular cloning and functional expression of rat and human 5-HT$_{1E}$-like 5-hydroxytryptamine receptor genes. Proc. Natl. Acad. Sci. USA *90*, 2184–2188.

Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M., and Julius, D. (1991). Primary structure and functional expression of the 5HT$_3$ receptor, a serotonin-gated ion channel. Science *254*, 432–437.

McAllister, G., Charlesworth, A., Snodin, C., Beer, M. S., Noble, A. J., Middlemiss, D. N., Iversen, L. L., and Whiting, P. (1992). Molecular cloning of a serotonin receptor from human brain (5HT$_{1E}$): a fifth 5HT1-like subtype. Proc. Natl. Acad. Sci. USA *89*, 5517–5521.

Medanic, M., and Gillette, M. U. (1992). Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker in vitro only during the subjective day. J. Physiol. *450*, 629–642.

Meijer, J. H., and Rietveld, W. J. (1989). Neurophysiology of the suprachiasmatic circadian pacemaker in rodents. Physiol. Rev. *69*, 671–707.

Monsma, F. J., Jr., Shen, Y., Ward, R. P., Hamblin, M. W., and Sibley, D. R. (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. Mol. Pharmacol. *43*, 320–327.

Pazos, A., Hoyer, D., and Palacios, J. M. (1985). The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. Eur. J. Pharmacol. *106*, 539–546.

Plassat, J.-L., Boschert, U., Amlaiky, N., and Hen, R. (1992). The mouse 5HT5 receptor reveals a remarkable heterogeneity within the 5HT1D receptor family. EMBO J. *11*, 4779–4786.

Pritchett, D. B., Bach, A. W. J., Wozny, M., Taleb, O., DalToso, R., Shih, J. C., and Seeburg, P. H. (1988). Structure and functional expression of cloned rat serotonin 5HT-2 receptor. EMBO J. *7*, 4135–4140.

Prosser, R. A., and Gillette, M. U. (1989). The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP. J. Neurosci. *9*, 1073–1081.

Prosser, R. A., Miller, J. D., and Heller, H. C. (1990). A serotonin agonist phase-shifts the circadian clock in the suprachiasmatic nuclei in vitro. Brain Res. *534*, 336–339.

Prosser, R. A., Dean, R. R., Edgar, D. M., Heller, H. C., and Miller, J. D. (1993). Serotonin and the mammalian circadian system. I. In vitro phase shifts by serotonergic agonists and antagonists. J. Biol. Rhythms *8*, 1–16.

Ralph, M. R., Foster, R. G., Davis, F. C., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975–978.

Rusak, B., and Zucker, I. (1979). Neural regulation of circadian rhythms. Physiol. Rev. *59*, 449–526.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.-L., and Hen, R. (1992). A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. EMBO J. *11*, 7–17.

Schwartz, W. J. (1993). A clinician's primer on the circadian clock: its localization, function, and resetting. Adv. Intern. Med. *38*, 81–106.

Shibata, S., Tsuneyoshi, A., Hamada, Y., Tominaga, K., and Watanabe, S. (1992). Phase-resetting effect of 8-OH-DPAT, a seroto-

nin$_{1A}$ receptor agonist, on the circadian rhythm of firing rate in the rat suprachiasmatic nuclei in vitro. Brain Res. *582*, 353–356.

Tominaga, K., Shibata, S., Ueki, S., and Watanabe, S. (1992). Effects of 5-HT$_{1A}$ receptor agonists on the circadian rhythm of wheel-running activity in hamsters. Eur. J. Pharmacol. *214*, 79–84.

Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A., and Hibert, M. (1992). Modeling of G protein–coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. J. Med. Chem. *35*, 3448–3462.

Voigt, M. M., Laurie, D. J., Seeburg, P. H., and Bach, A. (1991). Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. EMBO J. *10*, 4017–4023.

Witz, P., Amlaiky, N., Plassat, J.-L., Maroteaux, L., Borrelli, E., and Hen, R. (1990). Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. Proc. Natl. Acad. Sci. USA *87*, 8940–8944.

Zgombick, J. M., Weinshank, R. L., Macchi, M., Schechter, L. E., Branchek, T. A., and Hartig, P. R. (1991). Expression and pharmacological characterization of a canine 5-hydroxytryptamine$_{1D}$ receptor subtype. Mol. Pharmacol. *40*, 1036–1042.

Zifa, E., and Fillion, G. (1992). 5-hydroxytryptamine receptors. Pharmacol. Rev. *44*, 401–458.

GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is L22558.

International Clinical Psychopharmacology (1993), **8 Suppl. 2**, 19 - 23

Cloning of new 5-HT receptors

T.W. Lovenberg,[1] M.G. Erlander,[1] B.M. Baron[2] and J.G. Sutcliffe[1]

[1]*Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037 and* [2]*Marion Merrell Dow Research Institute, 2110 East Galbraith Rd, Cincinnati, OH 45215, USA*

Correspondence to: J.G. Sutcliffe at above address

INTRODUCTION

One of the goals of our research is the identification of genes associated with particular behaviors as a mechanism for understanding some of the biochemical processes that underlie those behaviors. We have a specific interest in genes that subserve hypothalamic function, particularly genes which may be involved in the control of endogenous circadian rhythms. As an initial approach to identifying genes expressed in the hypothalamus, we targeted the cloning of genes encoding receptors for the indoleamine neurotransmitters, serotonin (5-HT) and melatonin, both of which are known to affect hypothalamic function and also to play roles in the control of circadian rhythms (Miller and Fuller, 1990; Cassone, 1990).

At the outset of this work, genes encoding three G-protein-coupled 5-HT receptors (5-HT_{1A}, 5-HT_{1C}, and 5-HT_2) had been isolated by Kobilka *et al.* (1987), Lubbert *et al.* (1987), Fargin *et al.* (1988), Julius *et al.* (1988), and Pritchett *et al.* (1988). No gene encoding a melatonin receptor has yet been isolated, although there is a melatonin receptor in the suprachiasmatic nucleus (SCN) of the hypothalamus (Vanecek, 1988) and melatonin binding to its receptor has been shown to mediate pertussis toxin-sensitive inhibition of cAMP accumulation (Carlson *et al.*, 1989), suggesting that the receptor is a G-protein-coupled protein from the same protein superfamily. Since serotonin and melatonin are derived from tryptophan and have similar molecular structures, we reasoned that a study of receptor genes expressed within the hypothalamus and related to serotonin receptors might lead to identification of proteins involved in the mediation of hypothalamic indoleamine signaling.

All known G-protein-coupled receptors have a predicted topology that includes seven membrane-spanning domains which are highly conserved, particularly among members of various receptor subfamilies. Specific regions of these membrane-spanning domains contain the ligand (neurotransmitter) recognition sites. In 1989, Libert *et al.* demonstrated that, through the use of polymerase chain reaction (PCR), novel G-protein-coupled receptors could be amplified using degenerate primers corresponding to conserved regions of transmembrane domains (TMDs) III and VI.

To isolate clones of indoleamine-binding receptors belonging to the superfamily of G-protein-associated receptors, we have modified the approach originally described by Libert *et al.* (1989). We performed two sequential rounds of nested PCR on a rat hypothalamic cDNA template using degenerate primers in the second round of PCR that corresponded to conserved residues of indoleamine-binding receptors that were absent from other subtypes of receptors in this superfamily. This new strategy took advantage of the observation that 5-HT receptors are highly conserved in TMD V, but differ from other receptors in the same area. Thus, by designing one primer which was specific for the TMD V of 5-HT receptors, we increased the probability of isolating only indoleamine-binding receptors and decreased the probability of cloning cDNAs for other types of receptors. This strategy made it reasonable to characterize each novel receptor encountered with the expectation that its ligand was an indoleamine.

Employing two different variations of this refined strategy, we have isolated clones of four novel indoleamine-binding receptors originally designated as MR77, REC17, MR22 and REC20. All of these receptors turned out to be 5-HT receptors, each with a unique pharmacological profile and central nervous system distribution. These were subsequently named 5-HT_{1D}, 5-HT_{1A}, 5-HT_{5B}, and 5-HT_5, respectively. Of the four receptors, one (5-HT_5) is expressed most abundantly in the hypothalamus, where we have evidence that it plays a role in the serotonergic regulation of circadian rhythms.

STRATEGY

The specific design which led to the subsequent cloning of MR77, MR22 and REC17 from hypothalamic cDNA was a first round of PCR using degenerate primers corresponding to conserved regions in TMDs III and VI of both catecholamine and 5-HT receptors. This was followed by a second round of PCR using a degenerate primer corresponding to a conserved region of TMD V, specific only to 5-HT receptors, in conjunction with the same TMD III primer used in the first round of PCR (Fig. 1) (Lovenberg *et al.*, 1993a; Erlander *et al.*, 1993).

For isolating REC20, we performed the first round of PCR-mediated cDNA amplification using degenerate primers corresponding to regions of TMD III and TMD VII conserved among catecholamine and 5-HT receptors. The second PCR round used a degenerate primer corresponding to residues in TMD V specific to MR22 and REC17 in conjunction with the already-used TMD VII primer (Lovenberg *et al.*, 1993b).

SEQUENCE ANALYSIS

The PCR-derived cDNAs which resulted from these amplification strategies were used as probes to screen a rat hypothalamus cDNA library (for MR22, REC17 and REC20) or rat genomic library (for MR77) to obtain clones spanning the entire protein coding regions. The putative proteins encoded by the nucleotide sequences shared several amino acid sequence characteristics of members of the G-protein-coupled, seven-TMD receptor superfamily. A search of the protein and nucleic acid databases with the primary structures of these four putative receptors revealed that MR77 has significant amino acid sequence identity with known 5-HT receptors that are coupled to the inhibition of adenylate cyclase: human 5-HT_{1E} = 55%, dog 5-HT_{1D} = 48% and rat 5-HT_{1B} = 46%. In contrast, MR22, REC17 and REC20 have 30-35% amino acid sequence identity with catecholamine and 5-HT receptors alike, with none exhibiting overall dominant similarity. However, when specific segments of the TMDs were examined, particularly TMD V, the receptors more closely resembled 5-HT receptors than catecholamine or other receptors. These observations suggest that MR77 is a member of the 5-HT_{1E} subfamily, that MR22 and REC17, which have 68% mutual sequence identity form a new subfamily, and that REC20 falls into a subfamily by itself (Lovenberg *et al.*, 1993a,b; Erlander *et al.*, 1993).

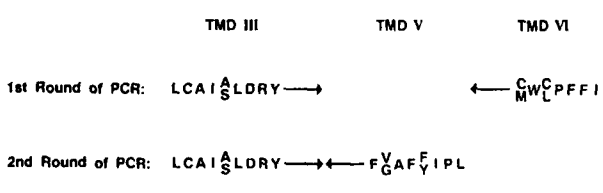

FIG. 1. Cloning strategy for identification of novel G-protein-coupled receptors. Oligonucleotides of degenerate sequences corresponding to all triplet codons for the amino acid sequences shown were synthesized. The sequences for TMD III and TMD VI are general for all biogenic amine receptors, whereas the sequence for TMD V is specific for 5-HT receptors.

MR22, REC17, MR77 AND REC20 ARE ALL 5-HT RECEPTORS

To determine the ligand(s) for these four putative receptors empirically, we subcloned their cDNAs into a eucaryotic expression vector and transiently expressed the encoded protein in CosM6 cells. We found that membranes prepared from transfected cells containing either MR22, REC17, MR77 or REC20 exhibited saturable binding for [^{125}I]-LSD whereas membranes from mock-transfected cells exhibited no measurable [^{125}I]-LSD binding (Lovenberg *et al.*, 1993a,b; Erlander *et al.*, 1993). ^{125}I-LSD has been shown previously to be a non-selective serotonergic ligand with high affinity (K_D < 10 nM) for all known 5-HT receptors except 5-HT_{1B} (K_D < 10-1000 nM; Peroutka, 1990). The calculated equilibrium dissociation constant (K_D) for the individual receptors is as follows: MR22 = 2.0 nM, REC17 = 0.64 nM, MR77 = 14 nM and REC20 = 1.5 nM. We next tested the ability of several biogenic amine neurotransmitters to displace [^{125}I]-LSD binding from these receptors. Serotonin was able to displace [^{125}I]-LSD binding to all receptors, whereas the neurohormones melatonin, dopamine and epinephrine had no measurable effects.

PHARMACOLOGICAL PROFILE AND CLASSIFICATION

To determine how each of these receptors related to known members of the 5-HT receptor family, we performed three analyses. First, we compared their primary structures with other known 5-HT receptors by a dendrogram analysis. Since we started this project, eight additional 5-HT receptors have been cloned, including three *Drosophila* receptors, 5-HTdrol, 5-HTdro2A and 5-HTdro2B (Voigt *et al.*, 1991; Zgombick *et al.*, 1991; Hamblin and Metcalf, 1991; McAllister *et al.*, 1992; Foguet *et al.*, 1992; Monsma *et al.*, 1993) (for review of current nomenclature see Humphrey *et al.*, 1993). The entire receptor family is depicted in Fig. 2. Second, we determined the ability of selective 5-HT agonists and antagonists to displace [^{125}I-]LSD from the membrane preparations. Third, we also expressed each receptor in HeLa cells to determine its ability to mediate cAMP accumulation.

MR77 = 5-HT_{1E}

[^{125}I-]LSD binding to membranes containing the MR77 protein is sensitive to sumatriptan, a 5-HT_{1D} agonist, but

insensitive to 5-carboxyamidotryptamine (5-CT), a mixed 5-$HT_{1A/1D}$ agonist, and 8-OH-DPAT, a 5-HT_{1A} agonist (Lovenberg *et al.*, 1993a). The pharmacological profile of MR77 is similar to that of the 5-HT_{1E} receptor. Because MR77 has greatest identity (55%) with a recently cloned 5-HT_{1E} receptor we conclude that we have identified a second member of the 5-HT_{1E} family and have designated MR77 as the 5-HT_{1F} receptor. This relationship is illustrated in the dendrogram shown in Fig. 2. As indicated, MR77 belongs to the general 5-HT_1 (5-$HT_{1A/B/D/E}$) family. In functional terms, we have demonstrated that MR77 (5-HT_{1F}) mediates the inhibition of cAMP accumulation in HeLa cells, presumably through a G_i-protein. This is also consistent with the findings that all other members of the 5-HT_1 family mediate the inhibition of adenylate cyclase. The mouse and human homologs of 5-HT_{1F} have also been identified (Amlaiky *et al.*, 1992; Adham *et al.*, 1993; Lovenberg *et al.*, 1993a).

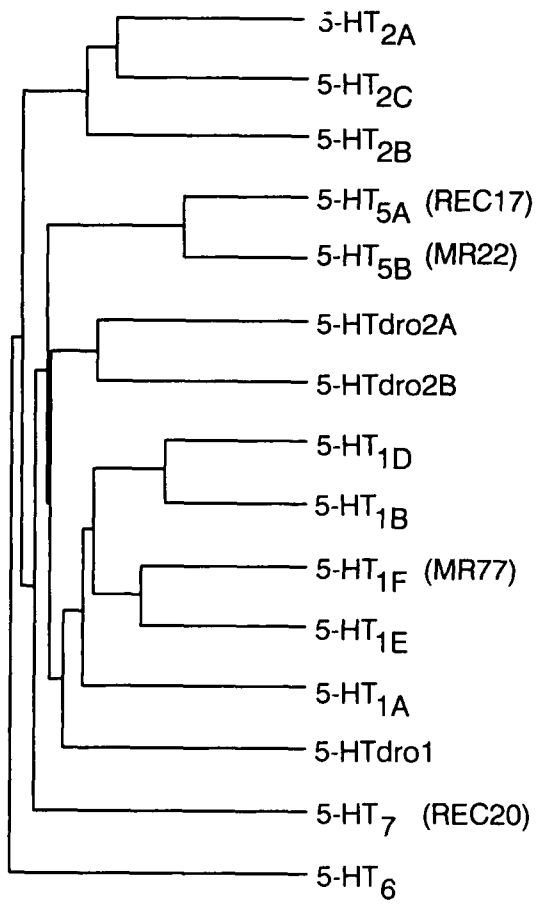

FIG. 2. Dendrogram of known 5-HT receptor subfamilies. This dendrogram represents the pairwise amino acid sequence comparisons of all 5-HT receptors. The lengths of the horizontal bars are inversely proportional to the percentage similarity of related sequences. For example, REC17 and MR22 are more similar to each other than any other pair of 5-HT receptors. REC20 and 5-HT_{1A} are sufficiently unique in sequence to form independent branches of the 5-HT family.

REC17 and MR22 are the 5-HT_{5A} and 5-HT_{5B} receptors

REC17 and MR22 have similar pharmacological profiles to one another (Erlander *et al.*, 1993) which do not readily fit to any pharmacologically defined 5-HT receptor profile. Both receptors are sensitive to ergotamine, 5-CT and methiothepin, but insensitive to sumatriptan and 8-OH-DPAT. This is consistent with their primary structural relationships with other known 5-HT receptors. As shown in the dendrogram, the primary structures of REC17 and MR22 lie in between those of the 5-HT_1 and 5-HT_2 (5-HT_{1C} and 5-HT_2) families. We are unable to link either REC17 or MR22 to a functional response such that neither receptor mediated stimulation or inhibition of cAMP accumulation in HeLa cells. Thus, REC17 and MR22 may couple to a second messenger other than cAMP. We propose that REC17 and MR22 are two members of a new subfamily of 5-HT receptors, designated as 5-HT_5. The mouse homologs of these two receptors have also been identified (Plassat *et al.*, 1992; Matthes *et al.*, 1992).

REC20 is the 5-HT_7 receptor

Comparison of the REC20 primary structure with other known 5-HT receptors indicates it forms a new subfamily (Fig. 2). In addition, REC20 has a pharmacological profile that is unique (Lovenberg *et al.*, 1993b). Displacement of [^{125}I]-LSD binding to REC20 is sensitive to not only the 5-HT_{1A} agonists 8-OH-DPAT and 5-CT but also sensitive to the 5-HT_2 antagonist ritanserin. Furthermore, REC20 binding is insensitive to the 5-HT_{1A} antagonist, pindolol. This unusual combination of agonist and antagonist affinities has provided a valuable means to discriminate REC20 function *in vitro* and *in vivo*. Functionally, REC20 stimulates the accumulation of cAMP in HeLa cells. Thus, unlike all other known 5-HT receptors, REC20 couples to a G_s-protein. Based on the novel functional and structural features of REC20, we classified it as a novel 5-HT receptor, 5-HT_7.

mRNA DISTRIBUTIONS

We performed Northern blots with rat poly(A)-selected RNA extracted from eight brain regions (cortex, hypothalamus, thalamus, hippocampus, striatum, pons, medulla and cerebellum) and heart, liver and kidney (Lovenberg *et al.*, 1993a,b; Erlander *et al.*, 1993). MR22 (5-HT_{5B}) mRNA was detected only in the hippocampal sample. REC17 (5-HT_{5A}) mRNAs were detected in hippocampus > cortex = thalamus = pons = striatum = medulla. REC20 (5-HT_7) mRNAs were found in hypothalamus = thalamus > pons = hippocampus. MR77 (5-HT_{1F}) mRNA was not detected in any of the tissues examined by

TABLE I. Characterization of novel 5-HT receptor clones

Clone	MR77	MR22	REC17	REC20
ORF	366	371	357	435
mRNA distribution	CX, STR, HIP HYP, THAL, PONS	HIP	HIP, CX, PONS, HYP STR, THAL, MED	HYP, THAL PONS, HIP
2nd Messenger	G_i	?	?	G_s
Sequence similarity	5-HT_{1E} (55%)	REC17 (68%)	MR22 (68%)	Unique (30% to all)
K_i (nM)	14	2	0.64	1.5
Pharmacological classification	5-HT_{1F}	5-HT_{5B}	5-HT_{5A}	5-HT_7

ORF refers to the triplet length of the open reading frame encoding the amino acid sequence. mRNA distribution was determined by Northern blot analysis (CX, cortex; STR, striatum; HIP, hippocampus; HYP, hypothalamus; THAL, thalamus; PONS, pons; MED, medulla). 2nd messenger coupling refers to the ability of the receptors to activate or inhibit adenylate cyclase through stimulatory (G_s) or inhibitory (G_i) G-proteins, respectively. The coupling of MR22 and REC17 is unknown (?). Sequence similarity was determined by comparison of the complete amino acid sequences to the receptors listed in the table. K_i values represent the concentration of [^{125}I]-LSD to half-saturate receptors transfected in CosM6 cells. The pharmacological classification indicates the names given to these clones within the 5-HT receptor family.

this method due to its low prevalence, however, by using a semi-quantitative PCR technique, we found MR77 mRNAs in cortex = striatum = hippocampus > thalamus = pons > hypothalamus > cerebellum. None of the receptor mRNAs was detected in heart, liver or kidney.

PHYSIOLOGICAL CORRELATES FOR RECEPTOR FUNCTION

Thus far, we have examined the mRNA expression patterns, pharmacological profiles and functional properties of each receptor. In Table I, we summarize these basic properties for MR77, MR22, REC17 and REC20. Since the goal of this research was to examine hypothalamic function through the identification of genes expressed in this region, we focused upon 5-HT_7 because it was the only receptor abundantly expressed in the hypothalamus. Additionally, we noticed that the agonists that activate 5-HT_7 are the same agonists that induce phase shifts in circadian electrical activity in SCN slices *in vitro* and *in vivo* when applied during subjective day (Medanic and Gillette, 1992; Prosser *et al.*, 1993; Edgar *et al.*, 1993). These effects have previously been thought to be mediated by 5-HT_{1A} receptors, however no 5-HT_{1A} receptor mRNA has been detected in the SCN (Roca *et al.*, 1992). In addition, it has been shown that an increase in the cAMP concentrations during the same window of time during subjective day results in a similar phase shift (Prosser and Gillette, 1989). 5-HT_7 is an attractive candidate for the relevant receptor because it binds these 5-HT_{1A} agonists, but unlike 5-HT_{1A}, is coupled to increases in cAMP. To discriminate between the 5-HT_7 and 5-HT_{1A} receptors we examined the effects of pindolol and ritanserin on the 5-HT-induced phase shifts. We found (Lovenberg *et al.*, 1993b) that the phase shifts were not blocked by pindolol, but were blocked by ritanserin. The agonist and antagonist profiles for the phase shift match those of the 5-HT_7 receptor, but not that of the 5-HT_{1A} or any other known receptor. These findings strongly suggest that 5-HT_7 is the receptor that mediates circadian phase advances during the subjective day.

CONCLUSION

We have presented here the description of four novel serotonin receptors. There are several aspects of this work that should be highlighted. At a basic level, this work demonstrates how structural information can be rationally used to identify new molecules at a genetic level. At a physiological level, we have demonstrated the existence of four serotonin receptors in the central nervous system. These discoveries should allow for a better understanding of how the brain serotonin system mediates behavioral phenomena. More specifically we have presented evidence implicating the 5-HT_7 receptor in the control of endogenous circadian activity of hypothalamic neurons. This will provide impetus for further study of the control of circadian rhythms by serotonin and may in fact provide a therapeutic target for manipulating mammalian circadian rhythms.

Acknowledgements

We thank our colleagues, whose collaborative studies we have reviewed here. This work was supported in part by AFOSR grant F49620-92-J-0188 and NIH grants NS22347 and GM32355.

REFERENCES

Adham N, Kao H-T, Schechter LE, Bard J, Olsen M, Urquhart D, Durkin M, Hartig PR, Weinshank RL and Branchek TA (1993) Cloning of another human serotonin receptor (5-HT_{1f}): A fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. USA*, **90**, 408-412.

Amlaiky N, Ramboz S, Boschert U, Plassat JL and Hen R (1992) Isolation of a mouse "5-HT_{1f}-like" serotonin receptor

expressed predominantly in the hippocampus. *J. Biol. Chem.*, **267**, 19761-19765.

Carlson LL, Weaver DR and Reppert SM (1989) Melatonin signal transduction in hamster brain: inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. *Endocrinology*, **125**, 2670-2676.

Cassone VM (1990) Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.*, **13**, 457-464.

Edgar DM, Miller JD, Prosser RA, Dean RR and Dement WC (1993) Serotonin and the mammalian circadian system. II. Phase-shifting rat behavioral rhythms with serotonergic agonists. *J. Biol. Rhythms*, **8**, 17-31.

Erlander MG, Lovenberg TW, Baron BM, De Lecea L, Danielson PE, Racke M, Slone AL, Siegel BW, Foye PE, Cannon K, Burns JE and Sutcliffe JG (1993) Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. *Proc. Natl. Acad. Sci. USA*, **90**, 3452-3456.

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MJ and Lefkowitz RJ (1988) The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature*, **335**, 358-360.

Foguet M, Hoyer D, Pardo LA, Parekh A, Fluxen FW, Kalkman HO, Stuhmer W and Lubbert H (1992) Cloning and functional characterization of the rat stomach fundus serotonin receptor. *EMBO J.*, **11**, 3481-3487.

Hamblin MW, and Metcalf MA (1991) Primary structure and functional characterization of a human 5-HT_{1D}-type serotonin receptor. *Mol. Pharmacol.*, **40**, 143-148.

Humphrey PPA, Hartig P and Hoyer D (1993) A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol. Sci.*, **14**, 233-236.

Julius D, MacDermott AB, Axel R and Jessell TM (1988) Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science*, **241**, 558-564.

Kobilka BK, Freille T, Collins S, Yang-Feng T, Kolbilka TS, Francke U, Lefkowitz RJ and Caron MG (1987) An intronless gene encoding a potential member of the family of receptors coupled to glanine nucleotide regulatory proteins. *Nature*, **329**, 75-79.

Libert F, Parmentier M, Lefort A, Dinsart C, Van Sande J, Maenhaut C, Simons M-J, Dumont JE and Vassart G (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science*, **244**, 569-572.

Lovenberg TW, Erlander MG, Baron BM, Racke M, Slone AL, Siegel BW, Craft CM, Burns JE, Danielson PE and Sutcliffe JG (1993a) Molecular cloning and functional expression of a novel rat 5-hydroxytryptamine receptor gene. *Proc. Natl. Acad. Sci. USA*, **90**, 2184-2188.

Lovenberg TW, Baron BM, De Lecea L, Miller JD, Prosser RA, Rea MA, Foye PE, Racke M, Slone AL, Siegel BW, Danielson PE, Sutcliffe JG and Erlander MG (1993b) A novel adenylate cyclase-activating serotonin receptor (5-HT$_7$) implicated in the regulation of mammalian circadian rhythms. *Neuron*, **11**, 449-458.

Lubbert H, Hoffman B, Snutch TP, Van Dyke T, Levine AJ, Hartig PR, Lester HA and Davidson N (1987) cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of mRNA-injected *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA*, **84**, 4332-4336.

Matthes H, Boschert U, Amlaiky N, Grailhe R, Plassat J-L, Muscatelli F, Mattei M-G and Hen R (1992) Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. *Mol. Pharmacol.*, **43**, 313-319.

McAllister G, Charlesworth A, Snodin C, Beer MS, Noble AJ, Middlemiss DN, Iversen LL and Whiting P (1992) Molecular cloning of a serotonin receptor from human brain (5-HT_{1F}): A fifth 5-HT_1-like subtype. *Proc. Natl. Acad. Sci. USA*, **89**, 5517-5521.

Medanic M and Gillette MU (1992) Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker *in vitro* only during the subjective day. *J. Physiol.*, **450**, 629-642.

Miller JD and Fuller CA (1990) The response of suprachiasmatic neurons of the rat hypothalamus to photic and serotonergic stimulation. *Brain Res.*, **515**, 155-162.

Monsma FJ, JR, Shen Y, Ward RP, Hamblin MW and Sibley DR (1993) Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol. Pharmacol.*, **43**, 320-327.

Plassat JL, Boschert U, Amlaiky N and Hen R (1992) The mouse 5-HT_{1D} receptor family. *EMBO J.*, **11**, 4779-4786.

Pritchett DB, Bach AWJ, Wozny M, Taleb O, Dal Toso R, Shih JC and Seeburg PH (1988) Structural and functional expression of a cloned rat serotonin 5-HT_2 receptor. *EMBO J.*, **7**, 4135-4140.

Prosser RA and Gillette MN (1989) The mammalian circadian clock in the suprachiasmatic nuclei is reset *in vitro* by cAMP. *J. Neurosci.*, **9**, 1073-1081.

Prosser RA, Dean RR, Edgar DM, Heller HC and Miller JD (1993) Serotonin and the mammalian circadian system. I. *In vitro* phase shifts by serotonergic agonists and antagonists. *J. Biol. Rhythms*, **8**, 1-16.

Roca AL, Weaver DR and Reppert SM (1992) Serotonin receptor gene expression in the rat suprachiasmatic nucleus. *Soc. Neurosci. Abs.*, **18**, 1221.

Vanecek J (1988) Melatonin binding sites. *J. Neurochem.*, **51**, 1436-1440.

Voigt MM, Laurie DJ, Seeburg PH and Bach A (1991) Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *EMBO J.*, **10**, 4017-4023.

Zgombick JM, Weinshank RL, Macchi M, Schechter LE, Branchek TA and Hartig PR (1991) Expression and pharmacological characterization of a canine 5-hydroxytryptamine$_{1D}$ receptor subtype. *Mol. Pharmacol.*, **40**, 1036-1042.

GENOMICS 19, 454–461 (1994)

Chromosomal Mapping of Mouse Genes Expressed Selectively within the Central Nervous System

PATRIA E. DANIELSON,* JOSEPH B. WATSON,† DAN D. GERENDASY,* MARK G. ERLANDER,* TIMOTHY W. LOVENBERG,* LUIS DE LECEA,* J. GREGOR SUTCLIFFE,*,[1] AND WAYNE N. FRANKEL‡

*The Scripps Research Institute, Department of Molecular Biology, La Jolla, California 92037; †Department of Psychiatry and Behavioral Sciences, Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, California 90024; and ‡The Jackson Laboratory, Bar Harbor, Maine 04609

Received July 20, 1993; revised October 29, 1993

We have used RFLP analysis on DNA from a panel of interspecific (C57BL/6J × *Mus spretus*) F_1 × *M. spretus* backcross offspring to assign the genes encoding 10 neuron-specific mRNAs and 2 loci corresponding to cyclophilin 2-related sequences to the mouse chromosomal map. The *Pss1* locus encoding the forebrain-enriched protein kinase C substrate RC3, a component of dendritic spines, mapped to proximal Chr 9. The *Camkl* locus encoding the calmodulin-binding protein kinase-like vesicle protein 1G5 mapped to distal Chr 9. The *Gng7* locus encoding the γ7 G-protein subunit, highly enriched in the striatum and presumptively coupled to dopamine receptors, mapped to mid-Chr 10. The *Htr1f*, *Htr5a*, *Htr5b*, and *Htr7* loci, encoding four serotonin receptors, mapped to Chr 16, 5, 1, and 19, respectively. The *Peplb* locus, encoding a CD26 ectopeptidase-like neuronal membrane protein activated by kainate and long-term potentiation, mapped to Chr 5. The *D2Sut1e* and *Cpu3* loci, encoding neural proteins of unknown functions, mapped to Chrs 2 and 9, respectively. Two cyclophilin 2-related loci, *Cphn2-r1* and *Cphn2-r2*, mapped to different regions of Chr 9. Comparison of these 12 newly mapped loci with the existing mouse map and known regions of syntenic homology with the human map, along with the known features and expression profiles of the products of these genes, suggests a few candidates for mouse mutations and human neurological and immunological deficits, including the Tourette syndrome and Bloom syndrome genes.

INTRODUCTION

The assembly of a database that includes the chromosomal location of each gene, the identity and nature of each gene product, and the anatomical and developmental windows of the product's expression will provide a firm foundation for understanding how the genome directs the formation and function of mammalian organisms. A substantial portion, perhaps as many as half, of mammalian genes are expressed selectively in the central nervous system (CNS), many with restricted expression within the CNS (Sutcliffe, 1988). We have pursued a systematic study to identify and elucidate the structures of mRNAs with selective expression within the rat CNS and the natures of their protein products. Here, we report chromosomal mapping studies of the mouse homologues of 10 novel genes encoding brain-enriched mRNAs and also the gene encoding cyclophilin 2. Our purpose is twofold. First, some insights as to the physiological functions of the genes might be gained were they to correspond to loci for which mouse neurological mutants or human neurological deficits had already been identified. Second, a thorough correlation of the genetic blueprint to its biochemical output is necessary for the full value of the Genome Project to be realized.

There are several defects in fairly ubiquitously expressed genes that manifest anatomically selective pathologies, such as the Lesch–Nyhan and Huntington diseases (Wu and Meltin, 1993; Huntington's Disease Collaborative Research Group, 1993). Thus, genetically determined selective pathology is not necessarily a result of mutation in a gene with restricted expression. However, mutations in genes whose expression is anatomically restricted are expected usually to give rise to focal pathologies, and examples abound. For instance, there are several defects in genes expressed specifically in the retina (*rds*, *rd*, opsin) that cause retinal degenerations or forms of blindness (Travis *et al.*, 1989; Farrar *et al.*, 1991; Kajiwara *et al.*, 1991, 1993; Nichols *et al.*, 1993; Wells *et al.*, 1993; Bowes *et al.*, 1990; Thaddeus *et al.*, 1990), and defects in the oligodendrocyte-specific genes *Mbp* and *Plp* lead to dysmyelination (Roach *et al.*, 1985; Nave *et al.*, 1987). Therefore, genes with restricted sites of expression merit consideration as candidates for diseases with corresponding focal pathologies. There will be exceptions; for example, a mutation in the pituitary-specific transcription factor Pit-1/GHF-1 causes a pa-

[1] To whom correspondence should be addressed. Telephone: (619) 554-8064. Fax: (619) 554-6112.

0888-7543/94 $6.00

thology involving multiple organ systems (Tatsumi *et al.*, 1992).

MATERIALS AND METHODS

Probe preparation. The inserts (usually full-length) of the cDNA clones (source references in Table 1) were released from their vectors by digestion with an appropriate restriction endonuclease and, after electrophoretic separation, were isolated from low-melting-point agarose gels by excision and heating. Aliquots (50 ng) were labeled by the random hexamer primer method (Feinberg and Vogelstein, 1983) and separated from unincorporated label by exclusion from Sephadex G-50. Salmon sperm DNA was added as carrier at 200 μg/ml. The probe was denatured by boiling and cooled on ice immediately before hybridization.

Restriction fragment length polymorphism (RFLP) detection. Genomic DNA samples (5 μg) from each parent and backcross individual (see below) were digested to completion with the restriction endonuclease *Eco*RI and separated by electrophoresis on 0.8% agarose/1× TBE gels run for 16–18 h at 1–1.5 V/cm. The gels were photographed and DNA nicked by ultraviolet irradiation, denatured in alkali, neutralized, and transferred by capillary action in 20× SSC to Biotrans nylon membranes (ICN, Irvine, CA). Filters were rinsed briefly in 20× SSC and cross-linked (120 mJ, total energy) in a Stratalinker 1800 (Stratagene, La Jolla, CA). Prehybridization, hybridization, and filter washing were performed as described (Southern, 1975), with the final wash at 68°C in 0.2× SSC, 0.2% SDS. Filters were exposed to Kodak XAR-5 film in the presence of two Dupont-Cronex Lightning Plus screens for 2–7 days at −70°C. After exposure, filters were stripped in boiling 5 m*M* potassium phosphate buffer (pH 7.0–7.5) for 10 min. Filters were reused 6–7 times. Approximate fragment sizes, scoring fragments, and probe sources are indicated in Table 1.

Genetic mapping and linkage analysis. The 11 cDNAs were mapped on a panel of 94 (C57BL/6J × SPRET/Ei) F_1 × SPRET/Ei interspecific backcross progeny that have been typed for over 500 genetic markers. This panel will be described in more detail in the future (E. Birkenmeier, L. B. Rowe, J. H. Nadeau, *et al.*, manuscript in preparation). The reference markers used for this study (Table 2; Fig. 1) were endogenous murine leukemia virus (MLV) integrations (*Mpmv*, *Pmv*, *Xmv*, *Mltr*, *Pltr*, and *Xltr* loci) and simple sequence length polymorphisms (SSLP; *D1Mit8*, etc). The chromosomal locations of these marker loci are known (Dietrich *et al.*, 1992; Frankel *et al.*, 1989a,b, 1990, 1991, 1992, 1993). The endogenous MLVs were typed by one of us (Frankel *et al.*, 1990), and the SSLP markers by Dr. J. H. Nadeau (pers. comm.) with the exceptions of *D9Mit8*, *D9Mit17*, *Trf*(*D9Mit24*), and *D10Mit8*, which were typed by us using the protocol of Dietrich and colleagues (1992).

The 11 cDNAs mapped to seven different mouse chromosomes each linked, with a high degree of confidence (LOD 9 or higher), to a known marker. The two-point linkage data are shown in Table 2, and multipoint crosses demonstrating gene order are shown in Fig. 1. Because five of the loci were on Chr 9, a linkage map for this chromosome is also provided (Fig. 2). The computer program RI Manager (Manly and Elliot, 1991) was used to assist in linkage analysis.

RESULTS AND DISCUSSION

To identify RFLPs that distinguish parental pairs, we digested genomic DNA from *Mus musculus* C57BL/6J (B6) and *Mus spretus* (SPRET) with the restriction endonuclease *Eco*RI. Southern blots prepared from the digested samples were probed with several of the cDNA clones listed in Table 1. For each probe examined, an *Eco*RI polymorphism was detected. Therefore, DNA from a panel of 94 (B6 × SPRET) F_1 × SPRET backcross offspring was digested with *Eco*RI, and the digested samples were used, along with digested parental samples, to prepare Southern blots. These were probed sequentially with the cDNA clones discussed below. All 11 cDNAs that we have examined to date exhibited *Eco*RI RFLPs (Table 1) that distinguished the two parental samples. These RFLPs were followed in the interspecific backcross panel to establish genetic linkage (Table 2, Figs. 1 and 2).

RC3: The Pss1 Locus on Chr 9

The brain-specific RC3 mRNA, highly enriched in cerebral cortex, striatum, and hippocampus, but absent from cerebellum, encodes a 78-amino-acid protein that is a substrate for protein kinase C and binds calmodulin in the absence of calcium (Watson *et al.*, 1990; Baudier *et al.*, 1991; Gerendasy *et al.*, 1992). The RC3 protein is found, both by immunoelectron microscopy and subcellular fractionation studies, to be highly concentrated in postsynaptic structures, particularly dendritic spines, in which neurochemical signaling related to protein kinase C and calmodulin-binding may occur (Watson *et al.*, 1992b; Coulter *et al.*, 1992). Consistent with its dendritic spine localization, RC3's postnatal-onset synthesis is severely and reversibly depressed by hypothyroidism, a condition known to decrease spine density (Munoz *et al.*, 1991; Ruiz-Marcos *et al.*, 1988). Accumulating evidence from biochemical and electrophysiological studies (Gerendasy *et al.*, 1992; Klann *et al.*, 1992; Cohen *et al.*, 1993) indicates that RC3 couples second-messenger systems to the processes of postsynaptic plasticity and long-term potentiation. RC3 has been independently discovered in several laboratories, where it is variously known as neurogranin (Baudier *et al.*, 1991), BICKS (Coggins *et al.*, 1991), P17 (Klann *et al.*, 1992), and clone 140 (Rhyner *et al.*, 1990).

The *Pss1* locus (for postsynaptic spine-*1* RC3 probe) mapped to Chr 9 and did not recombine in 94 progeny with *D9Mit2* (3.1-cM upper 95% confidence limit; Figs. 1 and 2, Table 2). Although there are no known neurological mouse mutations in this region of mouse Chr 9, *Pss1* is in a 12-cM region of linkage conservation with human Chr 11q22–q23, where ataxia telangiectasia complementation groups A, C, and D have been localized (Gatti *et al.*, 1988; Lambert *et al.*, 1991).

1G5: The Camkl Locus on Chr 9

The brain-specific, postnatal-onset 1G5 mRNA, enriched in forebrain and hypothalamus but absent from cerebellum, encodes a 504-amino-acid protein whose amino terminal 300 residues are highly similar to serine/threonine protein kinases, particularly (39% identity) Type II calcium/calmodulin-dependent protein kinase (Godbout *et al.*, 1993). The 1G5 protein is associated with membranes and vesicles in both axons and dendrites. Despite considerable sequence conservation with protein kinases, some critical ATP-binding residues are missing from 1G5, and recombinantly expressed 1G5 is inactive in protein kinase assays, although it binds calmodulin in the presence of calcium

TABLE 1

Locus	Probe[b]	Reference[c]	Fragment size (kb)[a] C57BL/6J[d]	Fragment size (kb)[a] *Mus spretus*
Pss1	RC3	Watson *et al.*, 1990	15, 12	15, 6.5
Camkl	1G5	Godbout *et al.*, 1993b	7	10
Gng7	CPu1 (γ7)	Watson *et al.*, 1993	6.0, 5.5[e], 3.0, 2.1	5.5[e], 3.0, 2.0
Htr1f	MR77 (5-HT_{1F})	Lovenberg *et al.*, 1993	19	3.5 (doublet)
Htr5a	REC17 (5-HT_{5A})	Erlander *et al.*, 1993	15, 6	15, 2.8
Htr5b	MR22 (5-HT_{5B})	Erlander *et al.*, 1993	20, 10, 5	20
Htr7	REC20 (5-HT_7)	Lovenberg *et al.*, 1993a	8, 5.5	8, 2.3
D2Sut1e	1B426b	Danielson *et al.*, 1989	16, 6	16
Peplb	BPL	de Lecea *et al.*, 1993	16	18
Cpu3	CPu3	Watson *et al.*, 1992a	18	12, 7.5
Cphn2-rs1	CPH2	Hasel *et al.*, 1991	20, 15, 8.5	20, 7.5
Cphn2-rs2	CPH2	Hasel *et al.*, 1991	20, 15, 8.5	20, 7.5

[a] Fragment sizes are approximations.
[b] All probes are rat cDNAs except CPu1 and CPH2, which are mouse.
[c] Origin of probe.
[d] Scoring fragments are underlined.
[e] Some strains contained a strongly hybridizing 5.5-kb fragment that was present in only about 10% of all backcross progeny. This fragment was not consistently found in all DNA samples from the same strain and, when present, its signal intensity seemed to vary inversely with the intensity of the 2.0-kb SPRET fragment. This fragment may represent unstable sequences associated with the *Gng7* locus or a related sequence.

(Godbout *et al.*, 1993). The accumulated evidence suggests that 1G5 is a vesicle- and membrane-associated protein whose (nonkinase) activity may have a serine/threonine specificity and be regulated by calcium/calmodulin.

Camkl (for calcium/calmodulin-dependent protein *k*inase-*l*ike 1G5 probe) mapped to distal Chr 9, 1.1 cM below transferrin (*Trf*), 1.1 cM proximal to the polytropic provirus *Pltr-6*, and 7.7 cM proximal to *D9Mit17* (Figs. 1 and 2; Table 2). Given the extensive linkage homology in this region of Chr 9, it is very likely that the human homologue of *Camkl* would reside on human Chr 3. In the mouse genome, *Camkl* is in the general vicinity of neurological mutations ducky (*du*), tippy (*tip*), and spinner (*sr*) (Green 1989).

Given the expression pattern of *Camkl* and its limitation to the CNS proper, we considered tippy the best candidate of the three mutations. Homozygous *tip/tip* mice are small and hyperactive and cannot stand or walk. We isolated DNA, poly(A)$^+$ RNA, and protein from *tip*$^-$ homozygotes and examined the samples by Southern, Northern, and Western blotting, using the 1G5 cDNA and 1G5 antisera as probes (as in Godbout *et al.*, 1993). We detected no differences between normal and *tip*$^-$ samples in terms of gene size or RNA/protein size or abundance (data not shown). Thus, the *tip*$^-$ allele is not an overt defect in the 1G5 gene.

γ7 G-Protein Subunit: The *Gng7* Locus on Chr 10

The postnatal-onset CPu1 mRNA, highly enriched in neurons of the caudate putamen, nucleus accumbens, and olfactory tubercule, encodes a 68-residue novel G-protein γ subunit, γ7 (Watson *et al.*, 1993; Cali *et al.*, 1992). The selective pattern of expression suggests that γ7 is a constituent of a heterotrimeric G-protein also containing $G_{olf\alpha}$, which couples a striatal dopamine receptor to the striatal-specific adenylate cyclase, AC_{ST} (Watson *et al.*, 1993).

Gng7 (for *G*-*n*ucleotide-binding protein *g*amma*7* subunit Cpu1 probe) mapped to mid-Chr 10, about 11.8 cM distal to *D10Mit5* and 7.4 cM proximal to *D10Mit8* (Table 2). This places it in the general vicinity of neurological mutations jittery (*ji*), Ames' waltzer (*av*), and mocha (*mh*). The human homologue of *Gng7* could reside either on human Chr 19p or 12q.

5-HT_{1F} Receptor: The *Htr1f* Locus on Chr 16

We have characterized four novel receptors for serotonin (5-HT) of the seven putative transmembrane receptor class. The 366-residue 5-HT_{1F} receptor (our clone MR77), coupled to inhibition of adenylate cyclase in response to serotonin, is expressed at low concentrations throughout the brain, with greatest expression in cerebral cortex, hippocampus, and striatum (Lovenberg *et al.*, 1993b; Adham *et al.*, 1993; Amlaiky *et al.*, 1992).

Htr1f (for 5-*HT* receptor *1f* MR77 probe) mapped to distal Chr 16, 7.9 cM proximal to the polytropic provirus *Pmv-14*. The only mouse mutation that resides in this region of the chromosome is dwarf (*dw*), which is the result of a mutation in the *Pit-1* gene (Camper *et al.*, 1990). Although the genetic map of mouse Chr 16 is rather sparse, the human homologue of *Htr1f* is likely to map to human Chr 3.

5-HT_{5A} Receptor: The *Htr5a* Locus on Chr 5

The 357-residue 5-HT_{5A} receptor (our clone REC17) is distributed throughout the CNS. Its closest known relative is the 5-HT_{5B} receptor (below). Second-messenger coupling is not known for either of these receptors (Erlander *et al.*, 1993; Plassat *et al.*, 1992).

TABLE 2

Chr	Interval	rec	Total	rf × 100	±SE (CI)	Lod
1	*D1Mit8-Htr5b*	11	93	11.8	3.4	13.3
	Htr5b-Mpmv-6	5	94	5.3	2.3	19.8
2	*Mpmv-3-D2Sutle*	8	91	8.8	3.0	15.6
	D2Sutle-Pmv-7	11	91	12.1	3.4	12.8
5	*D5Mit1-Peplb*	4	93	4.3	2.1	20.8
	Peplb-Xmv-45	0	94	0.0	(3.1)	28.3
	Htr5a-Peplb	0	62	0.0	(4.7)	18.7
	Xmv-45-Mpmv-7	6	94	6.4	2.5	18.6
9	*Cph2-rs1-D9Mit2*	17	94	18.1	4.0	9
	D9Mit2-Pss1	0	94	0.0	(3.1)	28.3
	Pss1-Xmv-16	2	94	2.1	1.5	24.1
	Xmv-16,D9Mit2l	3	92	3.3	1.9	22
	D9Mit21-Xmv-15	3	41	7.3	4.1	7.7
	Xmv-15-Cph2-rs2	1	39	2.6	2.5	9.7
	Cph2-rs2-D9Mit8	4	87	4.6	2.3	19.1
	D9Mit8-D9Mit24	17	94	18.1	4.0	9
	D9Mit24-Camkl	1	91	1.1	1.1	3.2
	Camkl-Pltr-6	1	91	1.1	1.1	25
	Pltr-6-D9Mit17	6	91	6.6	2.6	17.8
	D9Mit17-Cpu3	6	91	6.6	2.6	17.8
10	*D10Mit5-Gng7*	11	93	11.8	3.4	13.3
	Gng7-D10Mit10	7	94	7.5	2.7	17.5
16	*Htr1f-Pmv-14*	7	89	7.9	2.9	16.1
	Pmv-14-Pmv-16	7	94	7.5	2.7	17.5
19	*Htr7-Pltr-4*	0	90	0.0	(3.3)	27.1
	Pltr-4-D19Mit1	10	91	11.0	3.3	13.7
	D19Mit1-Xmv-18	5	91	5.5	2.4	19

Note. Two-point linkage data for cDNA loci are shown giving the number of recombinants (rec) between locus pairs, the total number of progeny tested, the recombination frequency × 100 (rf × 100), the standard error × 100 (±SE = $(rf(1 - rf)/total)^{0.5}$), or, in the case of no recombination, the 95% upper confidence limit (CI) obtained using a one-tailed test. The lod score for linkage was calculated according to Bishop (1985). Loci are listed from centromere to telomere.

Htr5a (for 5-*HT* receptor *5a* REC17 probe) was closely linked to *Peplb* (see below) on mouse Chr 5, with no recombinants in 62 backcross progeny (4.7-cM upper 95% confidence limit). This position is 3.3 cM distal to *D5Mit1* and 6.4 cM proximal to *Mpmv-7*. The only mouse neurological mutation in this region of the chromosome is reeler (*rl*), which is currently placed, however, more toward the centromere than *Htr5a* maps. *Htr5a* maps near the border of conserved homology with human Chr 7 and 4p16. The human holoprosencephaly-3 (HLP) gene maps to Chr 7q36 and might result from a serotonin receptor defect.

5-HT$_{5B}$ Receptor: The Htr5b Locus on Chr 1

The 371-residue 5-HT$_{5B}$ receptor (our clone MR22) is restricted to the CA1 region of the hippocampus, medial habenula, and raphe nuclei (Erlander *et al.*, 1993; Matthes *et al.*, 1993). Its second-messenger coupling is unknown.

Htr5b (for 5-*HT* receptor *5b* MR22 probe) mapped to mid-Chr 1, 11.8 cM distal to *D1Mit8* and 5.3 cM proximal to the endogenous provirus *Mpmv-6* (Table 2). This region is near the border of conserved linkage homology stretches with human Chr 1q and 2q. Interestingly, near this border, there is a small segment (so far defined only by the *Bcl-2* gene) that is homologous with human Chr 18q. This may be of interest because patients with the Gilles de la Tourette syndrome gene, GTS, which maps to human Chr 18q, have abnormal serotonin/platelet ratios (Comings, 1990). In addition, serotonin has been used successfully in the treatment of at least one GTS patient (Van Woert *et al.*, 1977). The *Htr5a* and *Htr5b* loci have recently been mapped by a chromosomal *in situ* hybridization method (Matthes *et al.*, 1993). The results obtained by the two approaches are in excellent agreement.

5-HT$_7$ Receptor: The Htr7 Locus on Chr 19

The 435-residue 5-HT$_7$ receptor (our clone REC20) is enriched primarily in thalamus and hypothalamus with moderate expression in cortex, hippocampus, and pons. This receptor is coupled to the stimulation of adenylate cyclase, and pharmacological evidence indicates that the 5-HT$_7$ receptor mediates serotonergic regulation of circadian rhythms in the hypothalamus (Lovenberg *et al.*, 1993a).

Htr7 (for 5-*HT* receptor 7 Rec20 probe) mapped to mid-Chr 19, with no recombinants in 90 backcross progeny (3.3-cM upper 95% confidence limit) with the polytropic provirus long terminal repeat *Pltr-4*. *Pltr-4* maps between *Cyp2c* and *Rbp-4* in BXD RI strains (W.N.F., unpublished results) and 11 cM proximal to *D19Mit1* (Table 2). This region of Chr 19 is homologous with human Chr 10q.

1B426b: The D2Sut1e Locus on Chr 2

The 1B426b gene utilizes two promoters and an alternative splice site to give rise to four mRNAs encoding four structurally distinct glycoproteins of 125, 153, 457, and 485 amino acids that share a 102-residue core sequence. The four gene products are detected in all regions of the rat brain (where their expression is restricted to neurons), anterior pituitary, and at extremely low concentrations in adrenal glands. Within brain, the mRNAs are especially enriched in cortex and hippocampus. The proteins are detected immunohistochemically in association with the cytoplasmic face of the rough endoplasmic reticulum and the cytoplasmic surfaces of vesicles within postsynaptic terminals (Danielson *et al.*, 1989; unpublished studies). No function for the 1B426b protein is known.

D2Sut1e (1B426b probe) mapped to proximal Chr 2, 8.8 cM distal to the endogenous provirus *Mpmv-3* and 12.1 cM proximal to the endogenous provirus *Pmv-7*. This location is near the border of conserved homology with human Chr 9q and Chr 2q.

BPL: The Peplb Locus on Chr 5

The brain-specific BPL mRNA, expressed widely in brain, but especially enriched in hippocampus, striatum, and medial habenula, encodes an 803-residue protein

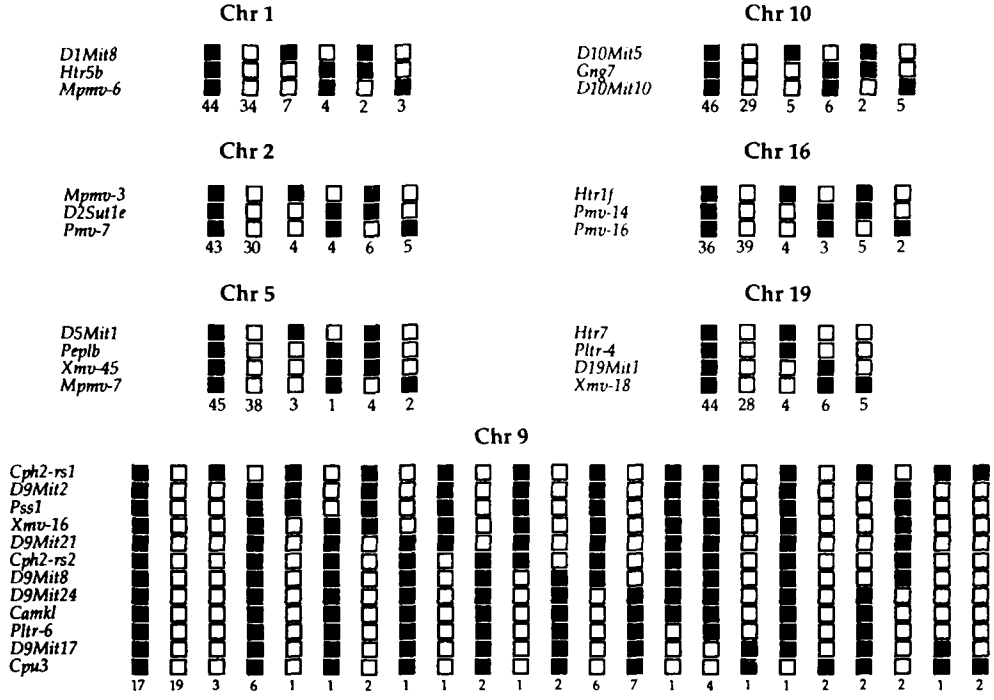

FIG. 1. Pedigrees of interspecific backcross progeny showing for each locus the inheritance of C57BL/6J alleles (black boxes) or SPRET/Ei alleles (white boxes) from the F_1, summed at the bottom of each column. A three-point analysis is shown for Chr 1, Chr 2, Chr 5, Chr 10, Chr 16, and Chr 19, and multipoint analysis is shown for Chr 9. *Xmv-15* (Chr 9) was omitted from the latter because it was typed in only half the progeny. Only progeny that were fully tested are shown.

that shares 30% identity with the membrane-anchored ectopeptidase CD26, which in T-cells binds to the specific tyrosine phosphatase CD45 (de Lecea *et al.*, 1993). The mRNA has been studied independently by Wada and colleagues (1992) and referred to as DPPX-S/L. Despite the considerable sequence identity with dipeptidyl peptidases, recombinant BPL demonstrates no peptidase activity, presumably due to the absence of a critical residue from its active site. Expression of the BPL mRNA in hippocampal neurons is stimulated several-fold by injection of the excitotoxin kainate and also in long-term potentiation (de Lecea *et al.*, 1993). No explicit model for BPL function has yet been proposed.

Peplb (for *pep*tidase-*l*ike *b*rain protein) mapped to proximal Chr 5, with no recombinants in 94 progeny (3.1-cM upper 95% confidence limit) with the endogenous xenotropic provirus *Xmv-45* or *Htr5a* (see above).

CPu3: The Cpu3 Locus on Chr 9

The CPu3 mRNA, brain specific with enrichment in striatum, pons, and medulla, but low in cerebellum, encodes a novel highly charged 81-amino-acid protein of unknown function (Watson *et al.*, 1992a; unpublished data).

Cpu3 (for *c*audate *pu*tamen-enriched *3*) mapped 6.6 cM distal to *D9Mit17*, probably quite near the telomere of Chr 9 (Fig. 2; Table 2). This region of the mouse genome is homologous with human Chr 3p.

Cyclophilin 2: Two Cphn2-rs Loci on Chr 9

The CPH2 mRNA, expressed ubiquitously in rodent tissues, encodes a 208-residue protein that shares 64% identity with cyclophilin 1. The CPH2 protein, which binds cyclosporin A and has cyclosporin A-inhibitable peptidyl-prolyl *cis–trans* isomerase activity, is associated with the endoplasmic reticulum (Hasel *et al.*, 1991; Price *et al.*, 1991).

The CPH2 cDNA probe for cyclophilin 2 hybridized with two distinctly segregating fragments. The first, *Cphn2-rs1* (for cyclophilin 2-related sequence *1*) mapped to proximal Chr 9, 18.1 cM proximal to *D9Mit2* (Table 2), placing it very near the centromere and the mouse mutation curly whiskers (*cw*), the "tail hair depletion" allele of which has an uncharacteristic immune response to certain polysaccharide antigens (Les and Roths, 1975; Roths, 1978). The second, *Cphn2-rs2* (for cyclophilin 2-related sequence 2), mapped in the middle of Chr 9, 4.6 cM proximal to *D9Mit8* and 2.6 cM distal to the endogenous provirus *Xmv-15*. This location makes the human homologue likely to be on Chr 15q, which may be of interest because Bloom syndrome, a dwarfism that is accompanied by severe immune defects, maps to Chr 15q26.1. The relationship between the two *Cphn2rs* loci and the true CPH2 structural gene is not yet known.

CONCLUSIONS

We have assigned the genes encoding 10 neuron-specific mRNAs and two cyclophilin 2-related loci to the mouse chromosomal map using RFLP analysis on a panel of 94 interspecific backcross mice. For 8 of these assignments, the probes hybridized to restriction fragments that were apparently identical between B6 and

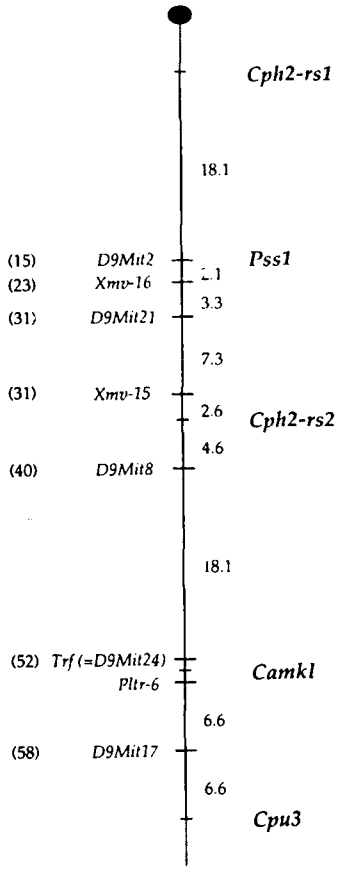

FIG. 2. Drawing of mouse Chr 9 linkage map showing positions of known markers on the left and those of newly mapped cDNAs on the right. The approximate positions of marker loci, based on a March 1993 consensus linkage map (Hillyard *et al.*, 1993) are shown in parentheses on the left. The sizes of the intervals between markers as determined from our interspecific cross are shown in recombination frequency units × 100.

SPRET in addition to the fragments exhibiting RFLPs (Table 1); thus, it will be important in the future to confirm the structure of each gene to ensure that, in each case, the true gene and not a pseudogene has been mapped. The size of the panel is sufficient to establish an average of 1- to 2-cM resolution. Syntenic regions on the human map have been identified for each locus, and candidate mutations and disease genes noted for most. Given what is known about the function and expression patterns of the proteins, the more attractive candidates seem to be GTS, *av*, and Bloom syndrome. Furthermore, given the highly focal expression pattern of the *Gng7* locus in caudate putamen and nucleus accumbens, it should be considered a candidate for any genetically determined movement or addiction disorder. Similarly, the restricted expression patterns of the *Pss1*, *Camkl*, *Htr5B*, *Htr7*, and *Peplb* loci should be factored when their candidate status for genetic diseases is evaluated.

The method we employed has a high success rate, but because of the time required for autoradiographic exposures and amount of DNA from the mapping panel required, it does not have the high throughput or efficiency that will be required to obtain map assignments for the 30,000 mRNAs expressed by the central nervous system (Sutcliffe, 1988). Ultimately, the construction of accurate physical maps and contigs of whole mammalian chromosomes, represented on hybridizable filters as yeast artificial chromosome inserts, will obviate the need for traditional genetic mapping of cloned cDNAs. Until such time, the methodology of single-stranded sequence polymorphism (Orita *et al.*, 1989ab), utilizing the polymerase chain reaction and primer pairs from the 3′ untranslated regions of the cDNA sequences, is more efficient than conventional hybridization for large-scale mapping and may be the method of choice for future chromosomal mapping of expressed sequences.

ACKNOWLEDGMENTS

We thank Drs. Ben Taylor and Janan Eppig for valuable comments on the manuscript, Dr. Ed Birkenmeier and Ms. Lucy Rowe for providing backcross DNAs, Dr. Joe Nadeau and Ms. Dean Turner for providing SSLP typings, and Dr. Verity A. Letts for typing MLV loci. W.N.F. is a Special Fellow of the Leukemia Society of America, Inc. This work was supported in part by grants from the NIH (NS22111, NS22347, GM32355, HG00332, and HD25831).

REFERENCES

Adham, N., Kao, H.-T., Schechter, L. E., Bard, J., Olsen, M., Urquhart, D., Durkin, M., Hartig, P. R., Weinshank, R. L., and Branchek, T. A. (1993). Cloning of another human serotonin receptor ($5\text{-}HT_{1F}$): A fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **90:** 408–412.

Amlaiky, N., Ramboz, S., Boschert, U., Plassat, J. L., and Hen, R. (1992). Isolation of a mouse "$5\text{-}HT_{1E}$-like" serotonin receptor expressed predominantly in the hippocampus. *J. Biol. Chem.* **267:** 19761–19765.

Baudier, J., Deloume, J. C., Van Dorsselaer, A., Black, D., and Matthes, H. W. D. (1991). Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). *J Biol. Chem.* **266:** 229–237.

Bishop, D. T. (1985). The information content of phase-known matings for ordering genetic loci. *Genet. Epidemiol.* **2:** 349–361.

Bowes, C., Li, T., Danciger, M., Baxter, L. C., Applebury, M. L., and Farber, D. B. (1990). Retinal degeneration in the *rd* mouse is caused by a defect in the β subunit of rod cGMP-phosphodiesterase. *Nature* **347:** 677–680.

Cali, J. J., Balcueva, E. A., Rybalkin, I., and Robishaw, J. D. (1992). Selective tissue distribution of G protein γ subunits, including a new form of the γ subunits identified by cDNA cloning. *J. Biol. Chem.* **267:** 24023–24027.

Camper, S. A., Saunders, T. L., Katz, R. W., and Reeves, R. H. (1990). The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics* **8:** 586–590.

Coggins, P. J., Stanisz, J., Nagy, A., and Zwiers, H. (1991). Identification of a calmodulin-binding, B-50-immunoreactive C-kinase substrate (Bicks) in bovine brain. *Neurosci. Res. Commun.* **8:** 49–56.

Cohen, R. W., Margulies, J. E., Coulter, P. M., II, and Watson, J. B. (1993). Functional consequences of expression of the neuron-specific, protein kinase C substrate RC3 (Neurogranin) in *Xenopus* Oocytes. *Brain Res.* **627:** 147–152.

Comings, D. E. (1990). Blood serotonin and tryptophan in Tourette syndrome. *Am. J. Med. Genet.* **36:** 418–430.

Coulter, P. M., II, Cohen, R. W., Margulies, J. E., and Watson, J. B. (1992). Assembly and PKC-phosphorylation of RC3 (neurogranin) in rat brain synaptosomes and voltage-clamped *Xenopus* oocytes. *Soc. Neurosci. Abstr.* **18:** 1345.

Danielson, P. E., Forss-Petter, S., Battenberg, E. L. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1989). A family of four structurally distinct neuron-specific glycoproteins produced by differential promoter utilization and alternative mRNA splicing from a single gene. *Abstr. Soc. Neurosci.* 451.10.

de Lecea, L., Soriano, E., Criado, J. R., Steffenson, S. C., Henriksen, S. J., and Sutcliffe, J. G. (1993). Transcripts encoding a neural membrane CD26 peptidase-like protein are stimulated by synaptic activity. Submitted.

Dietrich, W. F., Katz, H., Lincoln, S. E., Shin, H.-S., Friedman, J., Dracopoli, N., and Lander, E. S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics* **131:** 423–447.

Erlander, M. G., Lovenberg, T. W., Baron, B. M., de Lecea, L., Danielson, P. E., Racke, M., Slone, A. L., Siegel, B. W., Cannon, K., Burns, J. E., and Sutcliffe, J. G. (1993). Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. *Proc. Natl. Acad. Sci. USA* **90:** 3452–3456.

Farrar, G. J., Kenna, P., Jordan, S. A., Kumar-Singh, R., Humphries, M. M., Sharp, E. M., Sheils, D. M., and Humphries, P. (1991). A three-base-pair deletion in the peripherin-*RDS* gene in one form of retinitis pigmentosa. *Nature* **354:** 478–480.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction and endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.

Frankel, W. N. (1993). Endogenous proviruses mapped in recombinant inbred strains using long terminal repeat oligonucleotide probes. Submitted.

Frankel, W. N., Coffin, J. M., Cote, M., Seyfried, T. N., Rise, M., Rajan, T. V., Nelson, F. K., Selsing, E., and Gerstein, R. (1991). Backcross data for endogenous *Mpmv*, *Pmv*, and *Xmv* proviruses. *Mouse Genome* **89:** 266–270.

Frankel, W. N., Lee, B. K., Stoye, J. P., Coffin, J. M., and Eicher, E. M. (1992). Characterization of the endogenous nonecotropic murine leukemia viruses of NZB/BINJ and SM/J inbred strains. *Mamm. Genome* **2:** 110–122.

Frankel, W. N., Stoye, J. P., Taylor, B. A., and Coffin, J. M. (1989a). Genetic analysis of endogenous xenotropic MLVs: Association with two common mouse mutations and the viral restriction locus *Fv-1*. *J. Virol.* **63:** 1762–1774.

Frankel, W. N., Stoye, J. P., Taylor, B. A., and Coffin, J. M. (1989b). Genetic identification of endogenous polytropic proviruses by using recombinant inbred mice. *J. Virol.* **63:** 3810–3821.

Frankel, W. N., Stoye, J. P., Taylor, B. A., and Coffin, J. M. (1990). A genetic linkage map of endogenous murine leukemia proviruses. *Genetics* **124:** 221–236.

Gatti, R. A., Berkel, I., Boder, E., Braedt, G., Charmley, P., Concannon, P., Ersoy, R., Foroud, T., Jaspers, N. G. J., Lange, K., Lathrop, G. M., Leppert, M., Nakamura, Y., O'Connell, P., Paterson, M., Salser, W., Sanal, O., Silver, J., Sparkes, R. S., Susi, E., Weeks, D. E., Wei, S., White, R., and Yoder, F. (1988). Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. *Nature* **336:** 577–580.

Gerendasy, D. D., Wong, K., and Sutcliffe, J. G. (1992). Toward a function for the RC3 protein: A calmodulin-binding protein kinase C substrate located in dendritic spines. *Abstr. Soc. Neurosci.* 274.6.

Godbout, M., Erlander, M. G., Hasel, K. W., Danielson, P. E., Wong, K. K., Battenberg, E. L. F., Foye, P. E., Bloom, F. E., and Sutcliffe, J. G. (1993). 1G5: A calmodulin-binding, vesicle-associated, protein kinase-like protein enriched in forebrain neurites. *J. Neurosci.*, in press.

Green, M. C. (1989). Catalog of mutant genes and polymorphic loci. *In* "Genetic Variants and Strains of the Laboratory Mouse" (M. F. Lyon and A. G. Searle (Eds.), Oxford Univ. Press, Oxford.

Hasel, K. W., Glass, J. R., Godbout, M., and Sutcliffe, J. G. (1991). An endoplasmic reticulum-specific cyclophilin. *Mol. Cell. Biol.* **11:** 3484–3491.

Hillyard, A. L., Doolittle, D. P., Davisson, M. T., and Roderick, T. H. (1993). Locus map of mouse with comparative map points of human on mouse. The Jackson Laboratory, Bar Harbor, ME.

Huntington's Disease Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72:** 971–983.

Kajiwara, J., Hahn, L. B., Mukai, S., Travis, G. H., Berson, E. L., and Dryja, T. P. (1991). Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa. *Nature* **354:** 480–483.

Kajiwara, K., Sandberg, M. A., Berson, E. L., and Dryja, T. P. (1993). A *null* mutation in the human peripherin/RDS gene in a family with autosomal dominant retinitis punctata albescens. *Nature Genet.* **3:** 208–212.

Klann, E., Chen, S.-J., and Sweatt, J. D. (1992). Increased phosphorylation of a 17 kD protein kinase C substrate (P17) in long-term potentiation. *J. Neurochem.* **58:** 1576–1579.

Lambert, C., Schultz, R. A., Smith, M., Wagner-McPherson, C., McDaniel, L. D., Donlon, T., Stanbridge, E. J., and Friedberg, E. C. (1991). Functional complementation of ataxia-telangiectasia group D (AT-D) cells by microcell-mediated chromosome transfer and mapping of the AT-D locus to the region 11q22-23. *Proc. Natl. Acad. Sci. USA* **88:** 5907–5911.

Les, E. P., and Roths, J. B. (1975). *Mouse News Lett.* **53:** 34.

Lovenberg, T. W., Baron, B. M., de Lecea, L., Miller, J. D., Prosser, R. A., Rea, M. A., Foye, P. E., Racke, M., Stone, A. L., Siegel, B. W., Danielson, P. E., Sutcliffe, J. G., and Erlander, M. G. (1993a). A novel adenylate cyclase-activating serotonin receptor (5-HT_7) implicated in the regulation of mammalian circadian rhythms. *Neuron* **11:** 449–458.

Lovenberg, T. W., Erlander, M. G., Baron, B. M., Racke, M., Slone, A. L., Siegel, B. W., Craft, C. M., Burns, J. E., Danielson, P. E., and Sutcliffe, J. G. (1993b). Molecular cloning and functional expression of 5-HT_{1E}-like rat and human 5-hydroxytryptamine receptor genes. *Proc. Natl. Acad. Sci. USA* **90:** 2184–2188.

Manly, K. F., and Elliot, R. W. (1991). RI manager, a microcomputer program for analysis of data from recombinant inbred strains. *Mamm. Genome* **1:** 123–127.

Matthes, H., Boschert, U., Amlaiky, N., Grailhe, R., Plassat, J.-L., Muscatelli, F., Mattei, M.-G., and Hen, R. (1993). Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: Cloning, functional expression, and chromosomal localization. *Mol. Pharmacol.* **43:** 313–319.

Munoz, A., Rodriguez-Pena, A., Perez-Castillo, A., Ferreiro, B., Sutcliffe, J. G., and Bernal, J. (1991). Effects of neonatal hypothyroidism on rat brain gene expression. *Mol. Endocrinol.* **5:** 273–280.

Nave, K.-A., Bloom, F. E., and Milner, R. J. (1987). A single nucleotide difference in the gene for myelin proteolipid protein defines the jimpy mutation in the mouse. *J. Neurochem.* **49:** 1873–1877.

Nichols, B. E., Sheffield, V. C., Vandenburgh, K., Drack, A. V., Kimura, A. E., and Stone, E. M. (1993). Butterfly-shaped pigment dystrophy of the fovea caused by a point mutation in codon 167 of the RDS gene. *Nature Genet.* **3:** 202–207.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989a). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* **86:** 2766–2770.

Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989b). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5:** 874–879.

Plassat, J. L., Boschert, U., Amlaiky, N., and Hen, R. (1992). The mouse 5HT5 receptor reveals a remarkable heterogeneity within the 5HT1D receptor family. *EMBO J.* **11:** 4779–4786.

Price, E. R., Zydowsky, L. D., Jin, M., Baker, C. H., McKeon, F. D., and Walsh, C. T. (1991). Human cyclophilin B: a second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. *Proc. Natl. Acad. Sci. USA* **88:** 1903–1907.

Rhyner, T. A., Borbély, A. A., and Mallet, J. (1990). Molecular clon-

ing of forebrain mRNAs which are modulated by sleep deprivation. *Eur. J. Neurosci.* **2:** 1063–1073.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., and Hood, L. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* **42:** 149–155.

Roths, J. B. (1978). *Mouse News Lett.* **58:** 50.

Ruiz-Marcos, A., Cartagena, P., Garcia, A., Escobar del Rey, and Morreale de Escobar, G. (1988). Rapid effects of adult-onset hypothyroidism on dendritic spines of pyramidal cells of the rat cerebral cortex. *Exp. Brain Res.* **73:** 583–588.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503–517.

Sutcliffe, J. G. (1988). mRNA in the mammalian central nervous system. *Ann. Rev. Neurosci.* **11:** 157–198.

Tatsumi, K., Miyai, K., Notomi, T., Kaibe, K., Amino, N., Mizuno, Y., and Kohno, H. (1992). Cretinism with combined hormone deficiency caused by a mutation in the *PIT1* gene. *Nature Genet.* **1:** 56–58.

Thaddeus, P. D., McGee, T. L., Reichel, E., Hahn, L. B., Cowley, G. S., Yandell, D. W., Sandberg, M. A., and Berson, E. L. (1990). A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature* **343:** 364–366.

Travis, G. H., Brennan, M. B., Danielson, P. E., Kozak, C. A., and Sutcliffe, J. G. (1989). Identification of a photoreceptor-specific mRNA encoded by the gene responsible for retinal degeneration slow (*rds*). *Nature* **338:** 70–73.

Van Woert, M. H., Yip, L. C., and Balis, M. E. (1977). Purine phosphoribosyltransferase in Gilles de la Tourette syndrome. *N. Engl. J. Med.* **296:** 210–212.

Wada, K., Yokotani, N., Hunter, C., Doi, K., Wenthold, R. J., and Shimasaki, S. (1992). Differential expression of two distinct forms of mRNA encoding members of a dipeptidyl aminopeptidase family. *Proc. Natl. Acad. Sci. USA* **89:** 197–201.

Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1990). Subtractive cDNA cloning of RC3, a rodent cortex-enriched mRNA encoding a novel 78 residue protein. *J. Neurosci. Res.* **26:** 397–408.

Watson, J. B., Coulter, P. M., II, Margulies, J. E., de Lecea, L., Danielson, P. E., Foye, P. E., Erlander, M. G., and Sutcliffe, J. G. (1993). G-protein gamma-7 subunit is highly enriched in medium-size neurons of rat neostriatum. Submitted.

Watson, J. B., Coulter, P. M., II, and Sutcliffe, J. G. (1992a). Identification of genes enriched in expression in mammalian neostriatum and neocortex. *Brain Dysfunct.* **5:** 94–105.

Watson, J. B., Sutcliffe, J. G., and Fisher, R. S. (1992b). Localization of the PKC phosphorylation-calmodulin binding substrate RC3 in dendritic spines of neostriatal neurons. *Proc. Natl. Acad. Sci. USA* **89:** 8581–8585.

Wells, J., Wroblewski, J., Keen, J., Inglehearn, C., Jubb, C., Eckstein, A., Jay, M., Arden, G., Bhattacharya, S., Fitzke, F., and Bird, A. (1993). Mutations in the human retinal degeneration slow (*RDS*) gene can cause either retinitis pigmentosa or macular dystrophy. *Nature Genet.* **3:** 213–218.

Wu, C.-L., and Melton, D. W. (1993). Production of a model for Lesch–Nyhan syndrome in hypoxanthine phosphoribosyltransferase-deficient mice. *Nature Genet.* **3:** 235–240.