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THE ROLES OF NITRIC OXIDE, cGMP, AND cGMP-DEPENDENT PROTEIN KINASE IN GENERATION AND MODULATION OF CIRCADIAN RHYTHMS BY THE SUPRACHIASMATIC NUCLEUS

BY

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B.S., Slippery Rock University, 1988
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THE ROLES OF NITRIC OXIDE, cGMP, AND cGMP-DEPENDENT PROTEIN KINASE IN GENERATION AND MODULATION OF CIRCADIAN RHYTHMS BY THE SUPRACHIASMATIC NUCLEUS

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University of Illinois at Urbana-Champaign, 1995
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The hypothalamic suprachiasmatic nuclei (SCN) serve as the primary mammalian circadian pacemaker. While capable of generating near-24 hour rhythms in a variety of biochemical and behavioral rhythms, they can be entrained, or synchronized, to daily environmental cues such as light cycles. The neurobiology of neither the generation nor the modulation of circadian rhythms by the SCN is well understood.

Measurements from SCN in brain slice preparations from rats reveal a circadian rhythm of intracellular levels of the second messenger, cGMP, with peak levels late in a portion of the circadian cycle corresponding to nighttime in light/dark cycle of the donor animal (subjective nighttime). This late subjective nighttime rise in cGMP levels coincides with decreased levels of [γ-$^{32}PO_4$]-orthophosphate incorporation into a 75 kD protein in *in vitro* reactions stimulated by cGMP. This protein was identified as cGMP-dependent protein kinase (PKG) by immunoprecipitation and immunoblots using polyclonal antisera raised against this phosphoprotein extracted and purified from bovine brain. Relative levels of PKG over the circadian cycle were determined to be relatively constant. Thus, the decrease in incorporation is interpreted as a reflection of increased levels of endogenous autophosphorylation parallel to the endogenous rise in cGMP. The physiological significance of the endogenous rise in cGMP and PKG autophosphorylation is demonstrated

by *in vitro* assays of endogenous PKG activity in SCN from samples procured at different points in the circadian cycle. SCN samples from late subjective nighttime demonstrated significantly greater PKG activity at a time when endogenous cGMP levels and PKG autophosphorylation are highest.

In order to evaluate the role of nitric oxide (NO) in photic entrainment of the SCN, *in vivo* studies were performed in which wheel-running activity of hamsters under conditions void of photic information (constant darkness) was measured as an output of the circadian clock. The nitric oxide synthase inhibitor, L-NAME, was injected into the third ventrical at the level of the SCN, 10 minutes prior to photic stimulation and was shown to inhibit light-induced phase shifts of locomotor rhythms. The effect was reversible and dose-related, and could be outcompeted by addition of excess arginine, the substrate for NOS. The inactive isomer of the inhibitor, D-NAME, did not block photic phase-shifts. Neither L-NAME nor arginine affected phase of rhythms in the absence of light, nor did arginine potentiate light-induced phase shifts. However, while L-NAME did block phase shifts, it did *not* block light induced expression of the intermediate-early gene product, Fos, which has previously been correlated with and hypothesized to play a role in photic phase-shifting. Thus, it is concluded that NO is necessary for light-induced shifts in phase of the circadian oscillator, but that Fos production is either upstream from NO production or produced on a parallel light-input signal transduction pathway.

An attempt was made to measure transient increases in cGMP levels in the SCN following light stimulation. Radioimmunoassays for cGMP were performed on SCN samples procured from brains of hamsters microwave fixed at intervals following photic stimulation.

Results demonstrated that, while microwave fixation was rapid and efficient at halting enzymatic activity in brain areas assayed, measurements of cGMP and cAMP levels were highly variable, suggesting that this method is not suitable for cyclic nucleotide measurement.

Finally, the putative regulation of cAMP-response element binding protein (CREB) by PKG was evaluated using several approaches. CREBtide, a 14-amino acid sequence of CREB surrounding the activation-specific phosphoacceptor ser-133, was shown to be phosphorylated by PKG at a rate comparable to that of the PKG-specific heptapeptide substrate sequence of histone used to characterize PKG kinetics. [$^{32}PO_4$]-orthophosphate incorporation into a 43 kD protein corresponding to CREB on immunoblots was demonstrated in SCN in brain slices following subjective nighttime stimulation with a membrane permeable cGMP analog, 8-Br-cGMP, which has been previously shown to cause phase shifts in electrical activity rhythms in the SCN. Finally, specific phospho-CREB immunostaining in the SCN was significantly blocked by preincubation of the antiserum with CREBtide phosphorylated by PKG, further demonstrating the ability of PKG to phosphorylate CREBtide.

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I would like to thank my thesis committee members, Drs. Rhanor Gillette, Michael Glaser, Byron Kemper, and Tony Waldrop, for their insights and guidance. In addition, the lovely office personnel of the Physiology Department have made all the paperwork tolerable, and life in the department enjoyable.

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LIST OF ABBREVIATIONS

ACSF ..... artificial cerebrospinal fluid
APV ..... 2-amino-5-phosphonovaleric acid
BSA ..... bovine serum albumin
cAMP ..... adenosine 3',5'-cyclic monophosphate
CBP ..... CREB binding protein
cGMP ..... guanosine 3',5'-cyclic monophosphate
CRE ..... cAMP response element
CREB ..... cAMP response element binding protein
CT ..... circadian time
DD ..... dark/dark
EAA ..... excitatory amino acid
Fos-lir ..... Fos-like immunoreactivity
GABA ..... γ-aminobutyric acid
GHT ..... geniculohypothalamic tract
HEPES ..... *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
IEG ..... intermediate-early gene
IGL ..... intergeniculate leaflet
LD ..... light/dark
NAME ..... *N*-nitro-L-arginine methyl ester
NMDA ..... N-methyl-D-aspartate
NO ..... nitric oxide
NOS ..... nitric oxide synthase
PKA ..... cAMP-dependent protein kinase
PKC ..... protein kinase C
PKG ..... cGMP-dependent protein kinase
PKI ..... protein kinase inhibitor
$^{32}PO_4$ ..... [γ-^{32}P]-orthophosphate
PRC ..... phase response curve
PVDF ..... polyvinyl difluoride
R_1 ..... type-1 regulatory subunit (PKA)
RIA ..... radioimmunoassay
RHT ..... retinohypothalamic tract
SCN ..... suprachiasmatic nucleus
TCA ..... trichloroacetic acid

CHAPTER 1. SUBSTRATES OF THE CIRCADIAN SYSTEM: THE SUPRACHIASMATIC NUCLEUS, THE cGMP/PKG SYSTEM, AND PHOTIC REGULATORY PATHWAYS.

Throughout evolution, eukaryotic organisms have maintained organization of a variety of biochemical, physiological and behavioral activities to coincide with highly predictable daily events in the environment, the most notable of which is the rising and setting of the sun. This circadian (*"about a day"*) timekeeping system allows the organism to optimize performance of essential tasks such as food procurement and predator avoidance. In general, circadian timekeeping systems perform two functions: 1) generation of near-24 hour rhythms, and 2) modulation of those rhythms to maintain synchrony with the environment. This modulation allows for entrainment to environmental cues such as the solar cycle, and is accomplished through neural or hormonal signals which reset the phase of the pacemaker.

Research in this thesis addresses these two functions of the mammalian circadian pacemaker at a neurochemical level, examining the regulation of guanosine cyclic 3',5'-monophosphate (cGMP) and cGMP-dependent protein kinase (PKG) as components of the circadian pacemaker, and also investigating the roles of nitric oxide, cGMP, and PKG in transmitting environmental light cycle information to the pacemaker, possibly through regulation of the transcriptional factor, cAMP-response element binding protein (CREB).

The Suprachiasmatic Nucleus is the Primary Circadian Pacemaker

The primary circadian pacemaker in mammals is the suprachiasmatic nuclei (SCN) of the hypothalamus. The SCN generate approximate 24-hour physiological and behavioral rhythms (e.g., locomotor activity, eating and drinking, body temperature, and certain circulating hormone levels) under constant conditions, and may be entrained by

environmental time cues such as light (DeCoursey, 1964; Daan & Pittendrigh, 1976). Several pieces of evidence support the role of the SCN as the pacemaker. Electrolytic lesioning of the SCN results in loss of circadian organization of physiological and behavioral activities (Rusak, 1977; Inouye & Kawamura, 1979). Isolation of the SCN *in vivo* by surgically cutting around the SCN with a Halasz knife, thus severing neural communication, results in a loss of overt circadian rhythms in the animal, despite a continuing circadian rhythm of neuronal electrical activity in the SCN (Inouye & Kawamura, 1979). Robust circadian rhythms of spontaneous electrical activity (Green & Gillette, 1982; Prosser & Gillette, 1989), vasopressin secretion (Earnest & Sladek, 1987; Gillette & Reppert, 1987), and 2-deoxyglucose utilization (Newman & Hospod, 1986; Schwartz & Gainer, 1977; Schwartz *et al.* 1987; Shibata & Moore, 1988) persist in SCN in brain slices. A circadian rhythm of vasopressin secretion has also been demonstrated in dispersed cell culture (Murakami *et al.* 1991).

The most compelling evidence of SCN pacemaker function comes from transplantation experiments. Initial experiments demonstrated restoration of circadian organization in an SCN-lesioned animal following transplantation of donor SCN tissue (DeCoursey & Buggy, 1986; Lehman *et al.* 1987). Subsequent experiments involved transplantation of SCN tissue from tau (τ) period mutant hamsters, in which homozygous recessive individuals demonstrate 20 hour periods, into wild-type hamsters ($\tau = 24$ hours). Circadian rhythmicity was restored to the recipient with the period of the donor, proving that the components necessary for circadian timekeeping are contained in the donor SCN (Ralph *et al.* 1990).

The SCN are paired nuclei which have been characterized anatomically by cell morphology, neurotransmitter localization, and neural connections (van den Pol, 1980). In the rat, the nuclei have been subdivided into two regions, a dorsomedial region and a ventrolateral region, based on these characterizations. The dorsomedial region has small (6-8 μm diameter) neurons which may be arranged in chains (van den Pol, 1980) immunoreactive for vasopressin (Card *et al.* 1988) and neurophysin (van den Pol & Tsujimoto, 1985), and has been associated with output of the nucleus (Moore, 1983). The ventrolateral region has larger (8-11 μm diameter), loosely packed neurons, and is immunoreactive for vasoactive intestinal polypeptide (Card *et al.* 1988), neurotensin, gastrin releasing peptide, and bombesin (van den Pol & Tsujimoto, 1985). This region appears to receive most neural inputs to the nucleus (van den Pol, 1980).

Regulation of the Circadian Pacemaker

As the central component of a circadian timekeeping system, the SCN receive several major neural inputs. The SCN receive photic information via two pathways. The retinohypothalamic tract (RHT) contains direct projections from retinal ganglion cells which terminate primarily in the ventrolateral region of contralateral SCN (Moore & Lenn, 1972; Pickard, 1982; Pickard, 1985). RHT connections are necessary for entrainment to photic stimuli (Johnson *et al.* 1988a). Lesioning of all visual pathways except the RHT results in blindness but still allows for photic entrainment (Pickard, 1989). The putative primary neurotransmitter of optic tract is glutamate (Liou *et al.* 1986).

The geniculohypothalamic tract (GHT) originates in the intergeniculate leaflet (IGL) of the lateral geniculate nucleus in the rat, and projects primarily to ventrolateral regions of ipsilateral SCN (Card & Moore, 1988; Card & Moore, 1989). The GHT is not capable of entraining the SCN (Johnson *et al.* 1988a), and GHT-lesioned animals are capable of light entrainment through intact RHT (Johnson *et al.* 1989). The GHT is thought to convey information regarding light intensity (Zhang & Rusak, 1989), presumably through light intensity-sensitive type-W retinal ganglion cells (Groos & Meijer, 1985). The GHT is thought to utilize neuropeptide Y as its major transmitter (Card & Moore, 1989; Morin *et al.* 1992). The IGL also receives non-photic input from raphe nuclei, which may serve to regulate GHT input to the SCN.

The ventrolateral SCN also receives non-photic serotonergic projections from the dorsal and medial raphe nuclei (Aghajanian *et al.* 1969; van den Pol & Tsujimoto, 1985). These projections tend to synapse on common targets with neuropeptide Y projections from the GHT (Guy *et al.* 1987). The raphe nuclei also project directly to the intergeniculate leaflet (Moore *et al.* 1978), allowing the possibility of non-photic regulation of GHT projections to the SCN. Raphe projections have been associated with state of arousal, possibly regulating the SCN by pathways associated with motor activity of the animal, which, in rats and hamsters, is stimulated by onset of darkness (Mrosovsky & Salmon, 1987). Raphe projections are not necessary for photic entrainment of the SCN, and lesioning of this pathway does not impair SCN function (Smale *et al.* 1990).

Numerous studies have been performed in order to examine the effects of these pathways on SCN function. Effects on the SCN *in vivo* are often measured indirectly by

monitoring locomotor activity as an output of the clock, although other rhythms such as body temperature, drinking, and feeding are sometimes employed. Under conditions void of external circadian timing cues, these rhythms "free-run", or function with a circadian period intrinsic to their individual SCN, usually near but not exactly 24 hours. A relative method of timekeeping applied to circadian physiology employs the use of "circadian time" (CT) as 24 hours revolving around CT 12. CT 12 is defined as onset of activity, which, for nocturnally active animals such as rats and hamsters, corresponds to onset of darkness. For animals under a previous 12:12 hour light-dark cycle, the twelve hours prior to CT 12 are termed "subjective day" (corresponding to daytime in the prior lighting cycle), and the twelve hours after CT 12 are referred to as "subjective night." Thus, "circadian time" refers to timing relative to the phase of the circadian oscillator.

Particular stimuli elicit shifts in phase of SCN-generated rhythms only during restricted portions of the circadian cycle. These shifts in phase of the rhythms may be either advances or delays, and may be ascribed to the oscillating neurochemical state of the SCN. The relationship between (circadian) time of stimulation and effectiveness of the stimulus in shifting phase can be assessed at points throughout the circadian cycle, and thus periods of sensitivity of the SCN to particular stimuli may be described. This relationship is graphically represented as a phase response curve (PRC).

For example, animals housed under constant darkness are sensitive to light only during subjective nighttime. Light pulses delivered during early nighttime result in phase delays, while light pulses late at night induce phase advances. Light has no effect on phase of circadian rhythms during subjective daytime. Similar experiments were performed on

free-running animals in constant light, using brief periods of darkness as stimuli. While dark pulses also evoked both phase advances and delays, they did so during mid subjective day and early subjective night, respectively (Boulos & Rusak, 1982).

More invasive experiments such as direct electrical stimulation of tracts yield similar phase-response relationships. Direct electrical stimulation of the RHT at different times of the circadian cycle induces phase-shifts similar to those obtained with light pulses (Cahill & Menaker, 1989a). Alternatively, both stimulation of the GHT (Meijer *et al.* 1984) and stimulation of the SCN with neuropeptide Y by intracerebroventricular injections (Albers & Ferris, 1984) yield non-photic phase-shifts similar to dark pulses. The GHT projects to the SCN from the intergeniculate leaflet, and utilizes neuropeptide Y as a neurotransmitter (Card & Moore, 1982; Harrington & Rusak, 1985; Mason *et al.* 1987). IGL activity is modulated in part by serotonergic inputs from the midbrain raphe nuclei, the arousal center mediating signals of social interaction and activity/arousal state (Mrosovsky & Salmon, 1987; Mrosovsky, 1988; Mrosovsky *et al.* 1989; van Reeth & Turek, 1989).

The SCN in hypothalamic brain slices survive for several days in glucose-supplemented minimal salts solution and display electrical activity similar to that seen *in vivo* (Prosser & Gillette, 1989). Such isolation allows for experimentation on the steady-state pacemaker, and direct effects of various neuroactive agents on SCN function can be examined in the absence of modulatory inputs from other brain areas. Phase-response curves for serotonin (Medanic & Gillette, 1992), melatonin (McArthur *et al.* 1991), neuropeptide Y (Medanic & Gillette, 1993), glutamate ((Ding *et al.* 1994), nitric oxide

donors (Ding *et al.* 1994), and analogs of cAMP (Gillette & Prosser, 1988; Prosser & Gillette, 1989) and cGMP (Prosser *et al.* 1989) have been generated in this fashion.

While many neuroactive agents have been shown to affect the phase of SCN-generated rhythms, both *in vivo* and *in vitro*, little is known about the intracellular events that modulate these effects. Analogs of the cyclic nucleotide second messengers administered at particular points in the circadian cycle are capable of resetting the phase of the electrical activity rhythm in the SCN *in vitro* (Gillette & Prosser, 1988; Prosser & Gillette, 1989; Prosser *et al.* 1989). A cGMP analog advances the phase of the rhythm only when applied during subjective night. The coincidental overlap of temporal sensitivities of the SCN to light and cGMP suggest a possible mechanism by which effects of light input to the retina are conveyed into cellular signals involved in SCN entrainment.

Coincident with phase shifts in locomotor rhythms induced by light are characteristic patterns of immunoreactivity for the intermediate-early gene product, Fos, and its mRNA in the SCN (Rea, 1989b; Rusak *et al.* 1990; Rusak *et al.* 1992; Sutin & Kilduff, 1992). Fos, as well as several other intermediate-early gene products (e.g., Jun, NGF1A), can interact as transcriptional regulators. At times when animals are responsive to phase shifting by light, light stimulation also results in Fos-like immunoreactivity (Fos-lir) in the SCN. Fos-lir appears mainly in the ventrolateral regions, the target of a majority of RHT projections. This is evident at both delay and advance portions of the light-responsive period of the circadian cycle, with slight differences between these two times (Rea, 1992). No Fos-lir is evident in animals under constant dark conditions after photic stimulation during subjective daytime. Glutamate, a putative neurotransmitter of the optic nerves, has been implicated

in the Fos induction pathway, as inhibitors of both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors block photic induction of Fos (Abe *et al.* 1991; Abe *et al.* 1992; Rea *et al.* 1993a). This temporal coincidence in photic sensitivity has suggested a role for transcriptional regulation by Fos in light-entrainment models. However, no direct role for Fos has been elucidated, nor has the necessity of Fos for photic phase-shifting been demonstrated.

Recently, involvement of another transcriptional regulator, cAMP-response element binding protein (CREB), has been implicated in photic phase-shifting of the SCN. CREB immunoreactivity increases in the SCN following photic stimulation only during light-sensitive portions of the circadian cycle (Ginty *et al.* 1993). CREB is a 43 kD protein, with multiple phosphorylation sites for a variety of kinases (Ginty *et al.* 1993). Phosphorylation of at least one of these sites, ser-133, appears necessary for enhanced transcriptional activation through second messenger systems, and this serine is phosphorylatable by PKA (Lee & Masson, 1993).

Neurobiology of Glutamate

Glutamate is ubiquitously distributed throughout the mammalian brain, where it is present in higher concentrations than any other amino acid. It has been associated with a number of functions, the earliest being metabolic in nature (Kregs, 1935). It is an important building block of peptides and proteins, and it also serves as a precursor the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Recent studies have focused on the

powerful excitatory effects glutamate has on neurons; glutamate is now thought to be the most widely used excitatory neurotransmitter in the brain.

Glutamate receptors have been classified into at least four distinct categories based on agonist binding affinities and mechanism of action. The N-methyl-D-aspartate (NMDA) receptor is a Ca^{++}-permeant ion channel, requires glycine at low concentrations as a coagonist, and is antagonized by 2-amino-5-phosphonovaleric acid (APV) and MK-801. Non-NMDA ionotropic receptors have been further categorized into kainate receptors and AMPA/quisqualate receptors, both of which are associated with Na^+ and K^+ conductances upon glutamate binding, but which show preferential binding of agonists and antagonists. The fourth class of glutamate receptors are metabotropic, and are associated with G-proteins in the plasma membrane.

The putative neurotransmitter for the optic projections to the SCN is glutamate. Glutamate immunoreactivity has been localized to retinohypothalamic terminals in the SCN (Castel *et al.* 1993; deVries *et al.* 1993), and optic nerve stimulation releases [^{3}H]-glutamate and [^{3}H]-aspartate from hypothalamic explants containing the SCN (Liou *et al.* 1986). Antagonists of excitatory amino acid receptors block SCN responses to direct electrical stimulation of RHT in brain slices (Cahill & Menaker, 1987; Cahill & Menaker, 1989b) and to photic stimulation *in vivo* (Shibata *et al.* 1986; Ohi *et al.* 1991; Rea *et al.* 1992; Rea *et al.* 1993a). Several glutamate receptors subtypes have been localized to the SCN (Gannon & Rea, 1993; Gannon & Rea, 1994).

Characterization of cGMP-dependent Protein Kinase (PKG)

One mode of action of cyclic nucleotides is activation of cyclic nucleotide-dependent protein kinase pathways. Cyclic GMP-dependent protein kinase (PKG) has been localized to most mammalian tissues. Evolutionarily, PKG demonstrates similarities in sequence homology, cyclic nucleotide binding, and substrate specificity to cAMP-dependent protein kinase (PKA). However, differences in function and differential substrate specificities have been documented. While in many tissues, levels of PKA exceed PKG levels by 10- to 100-fold (Corbin & Lincoln, 1978), relatively high concentrations of PKG have been described in cerebellum (Lohmann & Walter, 1984), lung (Walter, 1981), smooth muscle tissue of vasculature and organs (Joyce *et al.* 1986), and platelets.

Two types of PKG have been characterized (types I and II). Cellular distribution of PKG between cytosol and membrane fractions is tissue-dependent, and may also be regulated within cells by molecular modifications such as phosphorylation. Type I exists as a homodimer and is mostly cytoplasmic (Lincoln & Corbin, 1983), while type II is monomeric, primarily membrane-bound, and found predominantly in intestinal epithelium (DeJonge, 1981).

Two isoforms of type I PKG (types Iα and Iβ) have been purified and differ only in sequences of their amino-termini (Wernet *et al.* 1989), which contain dimerization and autoinhibitory domains, as well as autophosphorylation sites. There are no significant differences in the catalytic domain or cGMP binding sites of the type I isoforms (Wolfe *et al.* 1989a), as reflected in similar substrate specificities and catalytic rates (Wolfe *et al.* 1989a). However, there are apparent differences in affinities for cGMP analogs, which may

be conferred by differences in amino terminus sequences. The two subtypes may also differ in post-translational modifications other than phosphorylation (Lincoln *et al.* 1988). Type Iα is predominant over type Iβ in cerebellum, lung, and organ smooth muscle, while more equal levels are observed in vascular smooth muscle (Wolfe *et al.* 1989a).

PKG is a serine/threonine kinase which contains dimerization, autoinhibitory, cGMP-binding, and catalytic (Mg-ATP binding and substrate-binding) domains. While PKG contains functional domains analogous to those within the PKA holoenzyme, unlike PKA, the regulatory and catalytic domains do not dissociate within PKG subunits, resulting in constant interactions within the enzyme (Fig. 1). This has made analysis of domain functions more difficult than for the dissociatable subunits of PKA.

The autoinhibitory domain is carboxy-terminal to the dimerization domain. Several autophosphorylation sites are present within the autoinhibitory domain. In type Iα PKG, the major autophosphorylated residue is thr-84, though ser-50, ser-72, and thr-58 are also autophosphorylatable (Aitken *et al.* 1984; Francis *et al.* 1992). In type Iβ, ser-63 is the major autophosphorylated residue, though others have not been conclusively demonstrated (Francis *et al.* 1992). Autophosphorylation of PKG increases more than four-fold upon binding of cGMP or cAMP, and acts to increase basal activity (Smith *et al.* 1992) as well as increase affinity for cAMP at the cyclic nucleotide-binding site (Hofmann *et al.* 1985b; Landgraf *et al.* 1986). The rate and duration of autophosphorylation is not inhibited by excess heptapeptide substrate, a sequence of histone specific for PKG (Glass & Krebs, 1982), suggesting that autophosphorylation is a preferential process over phosphorylation of exogenous substrates.

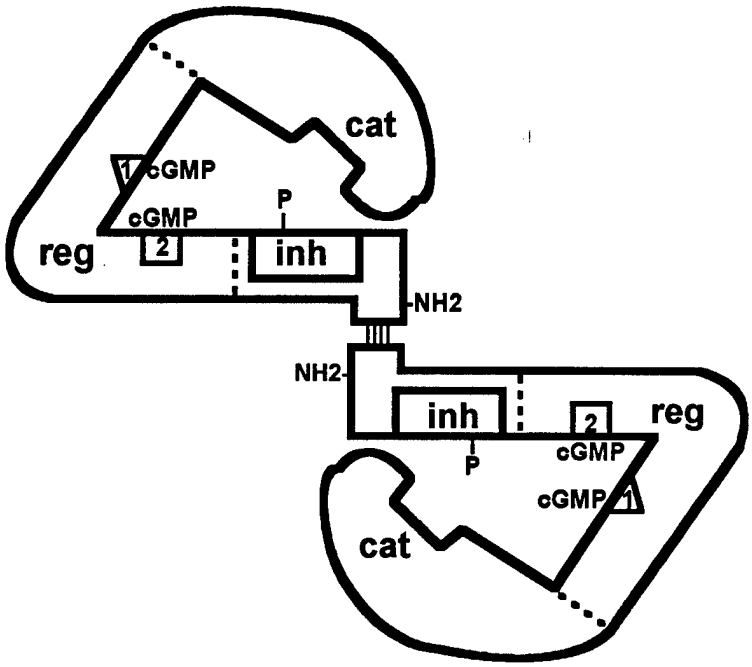


Fig. 1. Diagramatic representation of the PKG holoenzyme. cat= catalytic domain; reg= regulatory domain; 1= low-affinity cGMP-binding site; 2= high-affinity cGMP-binding site; inh= autoinhibitory domain, containing autophosphorylation sites (P); NH2-= amino-terminal dimerization site.

The physiological importance of autophosphorylation is not known. Experiments with cAMP-derivative stereoisomers (P*S*)-cAMP[S] and (P*R*)-cAMP[S] (Hofmann *et al.* 1985a) suggest that autophosphorylation regulates interaction between intra-subunit cGMP binding sites and the catalytic center, thus facilitating activation of the phosphotransferase reaction. An electronegative charge shift of PKG on a DEAE column has been demonstrated following binding of cGMP and/or autophosphorylation (Wolfe *et al.* 1987), suggesting that increased negative charge may repel elements in the catalytic domain which interact with the autoinhibitory domain, allowing increased enzyme activity. Alternatively, autophosphorylation could prolong duration of the activated state of the enzyme following cessation of a stimulus. This may occur not only because of increased affinity for cyclic nucleotides but also because of increased basal catalytic activity due to changes in configuration of the holoenzyme, even after release of cGMP, due to interference of binding between the autoinhibitory and the catalytic domains. The phosphorylated enzyme, because of a higher affinity for these ligands, would then be more responsive to small changes in cGMP or cAMP.

Each type I PKG subunit contains two cyclic nucleotide binding sites. Site 1 shows high affinity binding and relatively slower dissociation of cGMP and 8-Br-cGMP than site 2, with apparent positive cooperativity in binding (McCune & Gill, 1979; Wolfe *et al.* 1989a). While both sites bind cGMP and its analogs, site 1 also binds cAMP, while site 2 also binds cIMP (Corbin *et al.* 1986). These binding sites are located adjacent to the autoinhibitory domain on the subunit. Upon cGMP binding, inhibition of the catalytic domain is relieved, resulting in protein kinase activation (Wolfe *et al.* 1989b). Binding of cGMP to site 1

produces a partially activated enzyme, and subsequent binding to site 2 yields full activity. This positive cooperativity results in an expanded responsiveness over a broader range of cGMP concentrations (Corbin & Doskeland, 1983; Corbin *et al.* 1986).

The catalytic domain of PKG shares much structural similarity to the catalytic subunit of PKA. However, comparisons made between these enzymes are complicated by continual interaction of regulatory and catalytic domains on the same subunit of PKG, as opposed to individual separatable subunits of PKA. Furthermore, the endogenous protein kinase inhibitor (PKI) shows 100-fold greater affinity for PKA than for PKG. This specificity for some substrates and inhibitors is most likely imparted by distinguishing features of the larger catalytic domain of PKG. Both PKA and PKG preferentially phosphorylate substrates with the consensus sequence arg-arg-xxx-ser-xxx, but exceptions are documented (Glass & Krebs, 1979), most notably the autophosphorylation sites within type I PKG (Aitken *et al.* 1984; Smith *et al.* 1992), which lack the dibasic motif and contain a phospho-acceptor threonine instead of the more frequently phosphorylated serine residue. There is also strong selectivity by PKG versus PKA for phosphorylation sites on the cGMP-binding/cGMP-specific phosphodiesterase (Thomas *et al.* 1990; Colbran *et al.* 1992), PKA type-1 regulatory (R_1) subunit (Geahlen & Krebs, 1980), histone H1 (Flockerzi *et al.* 1978), and a 23 kD G-substrate isolated from mammalian cerebellar tissue (Aswad & Greengard, 1981; Schlichter *et al.* 1978). Analysis of several peptide substrates indicate that the presence of an aromatic residue near, and carboxy-terminal to, the phosphorylated serine/threonine is a negative determinant for PKA (Colbran *et al.* 1992). The structural features providing for this

selectivity are not clear, but probably reflect fine structural differences in the catalytic subunits of the two enzymes.

Neurobiology of Nitric Oxide

Glutamate has been associated with increases in cGMP in other brain areas (Mao *et al.* 1974; Ferrendelli *et al.* 1974; Danysz *et al.* 1989), many of which are transduced via a nitric oxide (NO) mechanism (Garthwaite *et al.* 1988; Bredt & Snyder, 1989; Knowles *et al.* 1989). Nitric oxide is a novel, putative neurotransmitter in the central nervous system. In nervous tissue, glutamate binds to NMDA receptors, leading to an increase in intracellular calcium. Elevated calcium levels result in calmodulin-mediated activation of nitric oxide synthase (NOS), an enzyme which is responsible for conversion of L-arginine to L-citrulline and NO (Miki *et al.* 1977). The enzyme requires $NADPH^+$ as a co-factor, and can be localized with an $NADPH^+$ diaphorase histochemical method (Vincent & Kimura, 1992). Several derivatives of arginine, including *N*-nitro-L-arginine, *N*-nitro-L-arginine methyl ester (L-NAME), and *N*-monomethyl L-arginine, bind to NOS but are not substrates for the enzyme, and thus serve as competitive inhibitors of that reaction (Palmer *et al.* 1988; Moore *et al.* 1990; Rees *et al.* 1990; Fukuto *et al.* 1990).

Under most physiological conditions, nitric oxide is an extremely labile molecule, either destroyed by free radical scavengers, or converted by oxygen and water to nitrates and nitrites. This results in a limited half-life estimated at approximately 4 seconds (Garthwaite, 1991). Nitric oxide is also bound avidly by heme-containing proteins such as hemoglobin and soluble guanylate cyclase (Bredt & Snyder, 1989). It is a lipid-soluble molecule capable

of diffusion through membranes, and once inside a neighboring cell, it can stimulate soluble guanylate cyclase, raising intracellular cGMP levels. Interestingly, guanylate cyclase does not appear to be stimulated in the cells in which NO is produced, presumably because it is inhibited by the increased intracellular calcium levels required for nitric oxide synthase activity (Mayer *et al.* 1992; Knowles *et al.* 1989).

The presence of nitric oxide synthase in the SCN was first suggested by a study demonstrating loss of autonomic responses to light following NOS inhibition (Amir, 1992). NOS has since been implicated in transduction of photic phase-shifting stimuli to the SCN in brain slices (Ding *et al.* 1994).

Foreward

The research described in chapters 2-4 herein address two functional properties of the suprachiasmatic nucleus: 1) the ability of the SCN to generate circadian rhythms, and 2) the ability of the SCN to shift the phase of those rhythms to coincide with environmental signals such as light cycles. Based on existing evidence from research on the SCN as well as from classical neurophysiological systems, the hypothetical model wherein glutamate from optic nerves stimulates nitric oxide synthase to produce nitric oxide, with subsequent production of cGMP and activation of PKG to regulate CREB and/or production of Fos, has been proposed and tested at numerous points.

In chapter 2, the involvement of cGMP and PKG in generation of circadian rhythms will be addressed, mainly by measurements of cGMP and PKG, as well as activity and phosphorylation state of PKG in SCN in brain slice, over the course of a circadian cycle.

In chapter 3, the elements of the model involving the roles of nitric oxide, cGMP, PKG, and Fos in photic resetting of the phase of SCN-generated rhythms *in vivo* are examined in experiments inhibiting nitric oxide production prior to photic phase-shifting and examining the resulting effects on phase of circadian locomotor rhythms and concommitant Fos production. Likewise, attempts to measure *in vivo* changes in cGMP levels following photic stimulation are described.

In chapter 4, the phosphorylation of the transcriptional activator, CREB, in the SCN via PKG is evaluated by examining changes, both *in vivo* and *in vitro*, in response to stimulation by two elements in the pathway. The results provide new insights into fundamental mechanisms in the mammalian biological clock.

CHAPTER 2. CIRCADIAN RHYTHMS IN THE cGMP-DEPENDENT PROTEIN KINASE SYSTEM IN THE RAT SUPRACHIASMATIC NUCLEUS.

This data is presently submitted for publication (Weber & Gillette, 1994).

Abstract

The primary circadian biological clock of mammals resides in the hypothalamic suprachiasmatic nuclei (SCN). In this series of experiments, several pieces of evidence point toward roles for guanosine 3',5'-cyclic monophosphate (cGMP) and cGMP-dependent protein kinase (PKG) in circadian timekeeping. 1) *In vitro* PKG activity measurements from suprachiasmatic nuclei free-running in brain slices from Long-Evans rats reveal a circadian change in endogenous basal activity, with a peak late during subjective night. 2) This change in basal activity coincides with changes in endogenous levels of cGMP over the circadian period. 3) *In vitro* endogenous phosphorylation in the suprachiasmatic nucleus in the presence of 200 μM cGMP shows variations in $^{32}PO_4$ incorporation from [γ-^{32}P]-ATP into PKG in the SCN; incorporation is lowest late at night. 4) Despite the changes in activity and $^{32}PO_4$ incorporation, the level of PKG remains constant over the circadian cycle, as determined by both activity assay and immunoblot. Taken together, the data suggest that endogenous changes in cGMP levels regulate PKG activity and phosphorylation state in timekeeping processes of this circadian clock.

Introduction

All eukaryotic organisms possess circadian clocks, which allow the organism to synchronize its biochemical, physiological and behavioral activities with environmental cycles. In general, circadian timekeeping systems are comprised of two components: 1) those which generate near-24 hour rhythms, and 2) those by which environmental signals modulate those rhythms. This modulation allows for entrainment of organismic activities

to environmental cues, the most powerful of which is the solar cycle, and is accomplished through neural or hormonal signals which reset the phase of the pacemaker.

The primary pacemaker for most, if not all, mammalian circadian rhythms is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. This has been demonstrated *in vivo* by lesioning (Rusak, 1977; Inouye & Kawamura, 1979), surgical isolation (Inouye & Kawamura, 1979) and transplantation experiments (DeCoursey & Buggy, 1986; Ralph *et al.* 1990; Lehman *et al.* 1987), and *in vitro* by demonstration of sustained electrical and biochemical rhythms in the brain slice (Green & Gillette, 1982; Gillette & Reppert, 1987; Prosser & Gillette, 1991; Newman *et al.* 1992) and in dispersed cell culture (Murakami *et al.* 1991).

Timing mechanisms can be examined in isolation from extrinsic neurochemical and neurohumoral agents by studying the SCN in the hypothalamic brain slice. The pacemaker survives for several days in glucose-supplemented minimal salts solution (Ding *et al.* 1994). Robust circadian rhythms of spontaneous electrical activity (Prosser & Gillette, 1989), vasopressin secretion (Earnest & Sladek, 1987; Gillette & Reppert, 1987), and 2-deoxyglucose utilization (Newman & Hospod, 1986; Schwartz & Gainer, 1977; Schwartz *et al.* 1987; Shibata & Moore, 1988) persist for several days under constant conditions *in vitro*, with the same phase relationship observed *in vivo*.

Many neuroactive substances have been shown to affect the phase of SCN-generated rhythms, both *in vivo* and *in vitro*, but little is known about the intracellular events which mediate these effects. Cyclic nucleotide analogs administered at temporally limited portions of the circadian cycle are capable of resetting the phase of the circadian rhythm of neuronal

activity in the SCN *in vitro* (Gillette & Prosser, 1988; Prosser & Gillette, 1989; Prosser *et al.* 1989). While adenosine 3',5'-cyclic monophosphate (cAMP) analogs act to advance the phase of the rhythm only if applied during subjective day (that portion of the circadian period analogous to daytime in the prior lighting schedule), guanosine 3',5'-cyclic monophosphate (cGMP) analogs advance the phase of the rhythm only during subjective night, and are ineffective during the daytime. Because the effect of each of these cyclic nucleotides is limited to a particular phase of the circadian cycle, biochemical substrates for some step(s) in each cyclic nucleotide pathway must be temporally restricted, and thus may serve as control points in the timekeeping process.

One might further hypothesize that these control points are stimulated by an endogenous change in these cyclic nucleotide effectors at an appropriate phase of the clock's cycle. It would follow that basal cGMP under unstimulated conditions should increase late in the period of sensitivity of the suprachiasmatic nuclei to phase-shifting by cGMP analogs. Thus, in order to further probe the role of cGMP in the suprachiasmatic nuclei, we examined basal levels of cGMP in brain slices throughout the circadian cycle *in vitro*. A circadian variation in endogenous levels of cGMP in unperturbed suprachiasmatic nuclei would imply a role either in rhythm generation or as an output of the circadian pacemaker. An increase in the levels of cGMP late during the period that the clock is sensitive to phase resetting by cGMP analogs would strengthen the hypothesis that it contributes to generation of circadian rhythms.

One mode of action of cyclic nucleotides is activation of cyclic nucleotide-dependent protein kinase pathways. Cyclic GMP-dependent protein kinase (PKG) is a

serine/threonine kinase which exists as a homodimer, with each subunit containing two cyclic nucleotide binding sites. The two intrachain binding sites display differences in binding kinetics and affinities for cGMP, as well as for other cyclic nucleotides and analogs. There appears to be a positive cooperativity in binding of cGMP (Corbin & Doskeland, 1983).

Binding of cGMP to PKG stimulates enzyme activity but is ineffective in stimulating autophosphorylation of PKG extracted from lung or heart tissue (Foster *et al.* 1981; de Jonge & Rosen, 1977). PKG is phosphorylatable at several serine and threonine sites within an autoinhibitory domain (Aitken *et al.* 1984), but the requirement of phosphorylation for kinase activity has not been conclusively demonstrated (Foster *et al.* 1981; Corbin & Doskeland, 1983). However, experiments using PKG from other sources suggest that autophosphorylation may regulate interaction between intra-subunit cGMP binding sites and the catalytic center (Wolfe *et al.* 1989b; Hofmann *et al.* 1985a).

The temporal restriction in efficacy of each cyclic nucleotide analog in phase resetting in the SCN clock could result from regulation either of PKG activity or of PKG levels. In order to evaluate the level of regulation cGMP levels were evaluated over the circadian cycle, PKG was identified in tissue punches of SCN, and changes in $^{32}PO_4$ incorporation, enzyme activity and relative levels of enzyme were assessed over the circadian cycle. Our results demonstrate that despite relatively constant levels of PKG, endogenous activity varies significantly over the circadian cycle. In addition, $^{32}PO_4$ incorporation into PKG during the day was significantly higher than that late at night, reflecting inverse differences in

endogenous state of phosphorylation over the circadian cycle. This suggests a mechanism by which PKG-induced changes in phase of the SCN are regulated.

Methods

Brain slice preparation

Hypothalamic brain slices were prepared from 7-8 week Long-Evans hooded rats raised in our inbred colony and maintained on a 12:12 light-dark cycle, as described previously (Prosser & Gillette, 1989). In the constant conditions of the brain-slice chamber, time is reckoned with respect to this entrained lighting cycle: circadian time (CT) starts with CT 0, equivalent to the time of lights-on in the colony, and continues for 24 hour although slices are in constant light. Slices were prepared only during subjective day, as slicing during the night results in phase shifts (Gillette, 1986). Slices were maintained in Earle's Balanced Salts Solution (GIBCO) supplemented with glucose (final 24.6 mM), sodium bicarbonate (26.2 mM), and gentamicin (0.005%), pH 7.4, 37°C. Up to 30 reduced slices were incubated together in a large volume brain slice chamber and allowed to equilibrate for at least 2 h. At desired time points, slices were removed from the chamber and quick-frozen (<20 seconds) on microscope slides on dry ice. The paired individual SCN were then punched out of each frozen slice with a frozen, modified 22 g needle (Palkovits, 1973) and stored either singly or pooled in Eppendorf tubes at -80°C.

cGMP measurement

At 4 ±0.5 hour intervals during the circadian cycle, samples of sixteen to twenty SCN punches were pooled for each time point. Individual assays included samples from each

time point. Each time point was repeated 4-8 times. Samples were stored at -80° C until time of assay.

Differences in cGMP content were examined at several circadian timepoints following varying lengths of incubation of the suprachiasmatic nuclei *in vitro*. As all samples within a group were obtained from animals of the same sex, differences in cGMP content between sexes were also examined at each circadian timepoint.

cGMP was extracted by sonicating the frozen tissue samples in 0.4 ml of cold 6% trichloroacetic acid (TCA) in a cooled immersion sonicator cup (Sonics and Materials, Danbury, CT). Samples were centrifuged at 4° C at 2000 *g* for 20 minutes (Beckman GPR). The supernatants were removed to conical glass centrifuge tubes and the TCA was extracted 4 times with 5 ml water-saturated ether. Recovery was calculated using a [^{3}H]-cGMP recovery assay. Samples were then lyophilized on a Savant Speed-Vac. Pellets from the TCA precipitation were assayed for protein after the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Supernatant residues were resuspended in 100 μl sodium acetate buffer (0.05 M, pH 6.2). Samples were assayed with a commercially available radioimmunoassay (RIA) kit (New England Nuclear), using the acetylation protocol. Counting was performed on a GammaTrac 1191 solid scintillation gamma counter (TMAnalytic, Inc.; 72% efficiency). Included in each assay were duplicates of three interassay standards prepared with the initial RIA. Cyclic nucleotide contents of each sample were computed with a log-logit transformation software program, corrected for aliquot removal and recovery, and normalized to unit protein content.

Results were analyzed by ANOVA. The Tukey-Kramer multiple comparison test was used to further describe differences among the time points. Length of incubation and sex differences in cGMP levels at several time points were analyzed using Student's t-test.

In vitro endogenous phosphorylation

Samples of single SCN were procured as described previously, at hourly intervals over the circadian period. For analysis of endogenous cGMP-dependent phosphorylation *in vitro*, punches were sonicated in 10 μl cold buffer containing 50mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM isobutylmethylxanthine (IBMX), 1 mM EGTA, 10 mM dithiothreitol, a cocktail of protease inhibitors (100 μM phenylmethulsulfonyl fluoride, 10 mM phenanthroline, 2 μM leupeptin, 0.2 μM pepstatin, 1% aprotinin; SIGMA), and 10 nM PKI (protein kinase A inhibitor; SIGMA). Reactions were initiated with addition of 10 μl reaction buffer containing 5 μM ATP, 3-5 μCi [γ-^{32}P]-ATP (NEN/Dupont), and 200 μM cGMP. After incubation for 2 minutes at 37° C, reactions were stopped with addition of 20 μl of 2X Laemmli buffer (Laemmli, 1970) and boiled for 3 minutes. Tissue was ruptured sufficiently upon freeze-thaw that subsequent sonication and/or grinding with a Teflon pestle did not affect the phosphoprotein profile observed in our gels.

Polyacrylamide gel electrophoresis

Samples were centrifuged at 10,000 *g* for 5 minutes. Supernatants were divided and separated by 1-D gel electrophoresis on both 8% and 15% SDS-polyacrylamide gels. Gels were then dried and exposed to X-ray film for 1-10 days. In some cases, gels were silver-stained prior to drying (Morrissey, 1981). Autoradiograms were quantitated with an LKB densitometer and analyzed using GelScan XL software. Area of peaks of interest were

normalized to total $^{32}PO_4$ incorporation for that sample. Averages of experiments were subjected to ANOVA, and differences were described further by Tukey multiple range test.

Polyclonal antibodies

Polyclonal antibodies were generated in order to identify and quantitate levels of the 75 kDa PKG substrate which showed variable $^{32}PO_4$ incorporation. The 75 kDa protein was affinity purified from bovine brain extract (Flockerzi *et al.* 1978) on a cAMP-derivatized Sephadex (SIGMA) column eluted with 1 mM cGMP (Wolfe *et al.* 1989b). Eluents were subjected to electrophoresis in low melting point 6% Prosieve agarose minigels (FMC Bioproducts). The 75 kDa band was then excised, melted and emulsified in phosphate-buffered saline and complete Freud's adjuvant (1:1) for initial subcutaneous injection into a rabbit (Sakakibara *et al.* 1987). The rabbit was boosted monthly thereafter using antigen emulsified in incomplete Freud's adjuvant.

Antibodies were characterized both by immunoprecipitation and immunoblotting, using commercially available PKG (Promega) as a substrate (described below).

Immunoprecipitations

In order to further characterize the specificity of the polyclonal antibodies, bead subtraction experiments were performed first on aliquots of commercially purchased PKG and then on SCN tissue homogenates following *in vitro* endogenous phosphorylation reactions. As it could not be determined whether several other small bands extracted by the antibodies in preliminary experiments were separate proteins or were breakdown products of PKG, in subsequent reactions, commercial PKG was diluted with buffer containing 1 mg/ml BSA in order to help maintain structural integrity. Phosphorylation

reactions were performed as previously described except that they were terminated by boiling, without addition of Laemmli buffer. Samples were then incubated with the presumptive anti-PKG polyclonal antibodies (1:500), 45 min, 4°C. In half of the samples, antibodies were preincubated with cold hypothalamic homogenate or commercial PKG. Following incubation, 100 μl of 50% (v/v) slurry of Protein A-derivatized sepharose beads (SIGMA)/buffer preabsorbed with hypothalamic homogenate, was added for 30 minutes at 4°C, shaking every 10 minutes. Samples were then centrifuged at 10,000 *g*, 5 min, 4°C. Supernatants were removed, diluted 1:2 with 2X Laemmli buffer and boiled 3 minutes. Bead fractions were washed 3X with 500 μl buffer, then boiled 3 minutes in 50 μl 2X Laemmli buffer. All fractions were then centrifuged 10,000 *g*, and supernatants were subjected to SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to X-ray film.

Immunoblotting

In order to determine whether changes in phosphorylation were due to changes in the amount of enzyme, samples containing either 6 pooled SCN or aliquots of commercially purchased PKG were subjected to *in vitro* endogenous phosphorylation reactions as described above and separated on 8% SDS-polyacrylamide mini-gels. Proteins were then electrotransferred (70V constant, 1.5 hours) to PVDF membrane (Immobilon-P, Millipore). Transfer buffer contained 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol and 0.05% SDS. Membranes were then immunoblotted at room temperature as follows: (a) overnight block in 5% Carnation powdered milk in T_{50}BS (50mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% thimerosol); (b) 1 hour incubation in same buffer with 0.05% TWEEN-20; (c) 1.5

hour incubation with polyclonal anti-PKG antibody (1:500 in T_{50}BS + 1% milk); (d) 10 minutes wash with T_{50}BS and 0.05% TWEEN-20 at 37%, repeated 3X; (e) 1 hour incubation with goat-anti-rabbit HRP-linked IgG in T_{50}BS + 1% milk; (f) 5 minutes wash with T_{50}BS without TWEEN-20, repeated 2X. At this point, the blot was developed with Amersham ECL bioluminescence system, then exposed to X-ray film for ^{32}P-labelled band comparison to the immunostaining results. Results were quantitated by 2D laser densitometry. Optical absorbances were normalized to CT 10 values within blots and were subjected to Student's t-test.

PKG activity assay

Activity of PKG was measured in individual SCN punches sampled at CTs 7, 18 and 23, by a method modified from Wolfe *et al.* (1989) using the PKG-specific heptapeptide substrate, arg-lys-arg-ser-arg-ala-glu (Peninsula Labs). Buffers were supplemented with protease inhibitor cocktail (see *"In vitro endogenous phosphorylation"*), 0.1 mg/ml BSA, 10 μM glycerophosphate as an excess substrate for phosphatases, 0.1 μM KT-5720 (PKA inhibitor; Kamiya Biomedical Co.), 1-2 μCi [γ-^{32}P]-ATP and 5 μM unlabelled ATP. Stimulated reactions contained 200 μM 8-Br-cGMP. Inhibited reactions contained 0.5 μM H-89 (Calbiochem), an inhibitor of PKG, in order to demonstrate additionally the specificity of cGMP-dependent stimulation. Reactions were carried out for 2 minutes at 37°C, then stopped by addition of 10 μl of 0.5 N HCl and spotting of 25 μl aliquots onto Whatman P-81 disks, which were then washed three times with 0.5% phosphoric acid, once with ethanol, dried and counted. Averages among CT groups as well as among treatments for each CT

group were subjected to ANOVA, and significant differences were further described by Tukey multiple range tests.

Results

I. Cyclic nucleotide levels

Assay validation

The protocol for the RIA kit generated a high rate of recovery of cGMP from the tissue and an acceptable range of variation between assays. Tissue extraction procedures yielded approximate 88.8% recovery of cGMP, as determined by [^{3}H]-cGMP recovery assay. Data were corrected for percent recovery. Variation within the cyclic GMP RIA ranged from 2.3% at standard curve midrange, to 3.6% at extremes of the curve. Interassay variation ranged from 10.9% at assay midrange, to 12.7% at extremes. Sample sizes were designed to fall within 30-70% binding range of the assay.

Basal cyclic GMP

Changes in cyclic GMP levels in SCN over a 31-hour period are shown in Fig. 2. Basal cyclic GMP levels ranged from 0.399 to 0.605 pmol cyclic GMP/mg protein (Table 1). Of the total data points (n=42), three statistical outliers (values greater than three standard deviations from the mean) were excluded from analysis. Statistical evaluation indicated a significant change in cGMP levels over the circadian period (ANOVA, $p<0.05$). Furtherpair-wise analysis with the Tukey-Kramer procedure indicated a significant difference between CT 11 and CT 23 ($p<.05$). The CT 7 time point assayed on day 2 *in vitro* was not

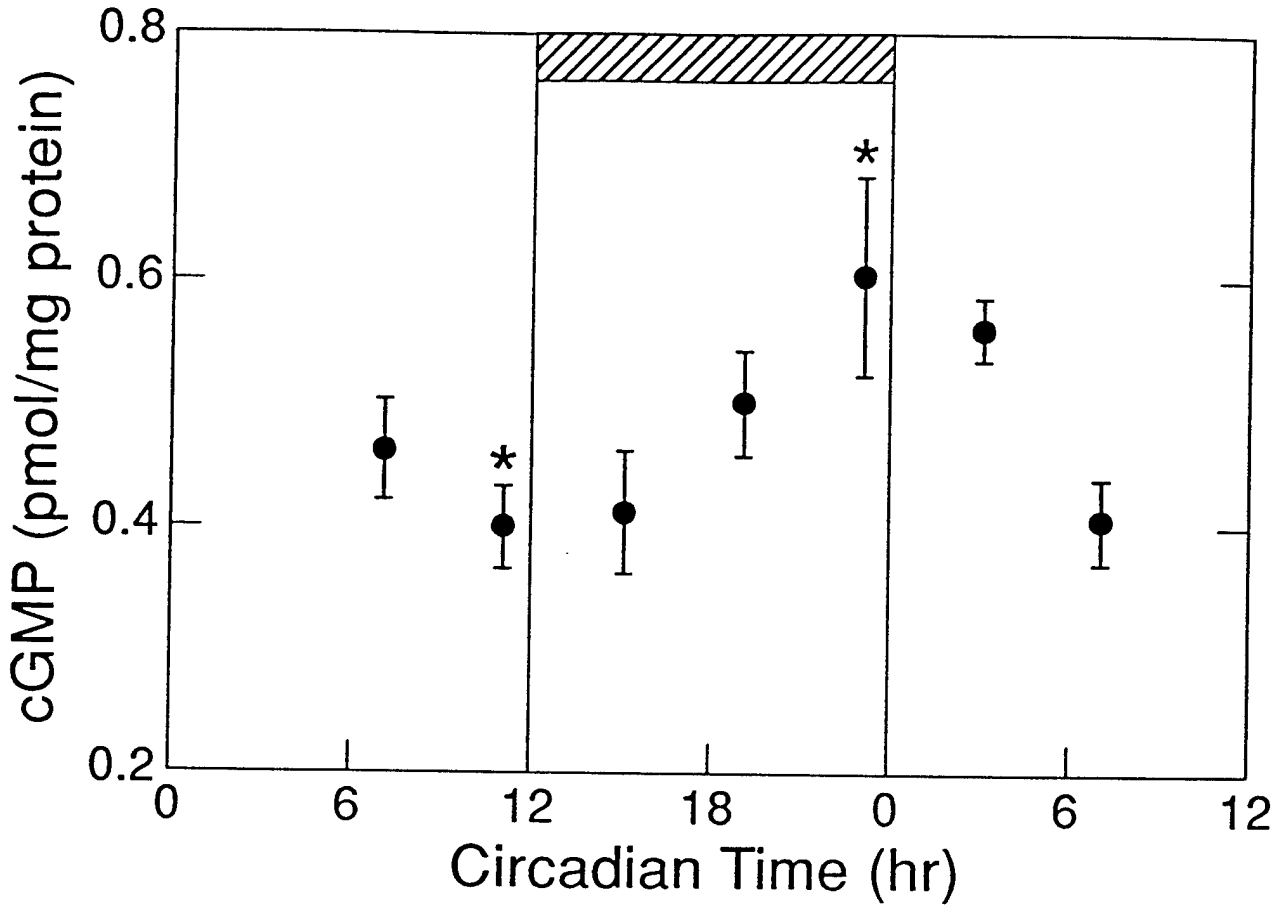


Fig. 2. Levels of endogenous cGMP in the SCN vary significantly over the circadian period. Peaks levels are at CT 23 and lowest levels are at CT 11.

statistically different from day 1 *in vitro* (Student's t-test, $p>.05$; Fig. 2), demonstrating a continuing circadian rhythm in cyclic GMP in the SCN in the brain slice.

Table 1. Basal levels of endogenous cyclic GMP in the SCN vary over the circadian cycle[1].

CT (hr)	cGMP (pmol/mg protein)
3	0.506 ± 0.054
7	0.462 ± 0.104 #
11	0.399 ± 0.096*
15	0.411 ± 0.125
19	0.501 ± 0.107
23	0.605 ± 0.225*
7, day 2	0.407 ± 0.067 #

[1] Statistically significant differences in cGMP levels in the SCN as measured by radioimmunoassay are evident between samples from late subjective day and late subjective night (*, Tukey-Kramer multiple range test, $p<.05$). Levels of cGMP at CT 7 on days 1 and 2 *in vitro* were not different (#, Student's t-test, $p>.05$).*Sex differences*

No statistical differences were seen between sexes for cyclic GMP in the SCN ($p>.05$). In addition, neither sex had consistently higher or lower levels of cGMP over the course of the circadian oscillation.

II. PKG regulation

Analysis of *in vitro* endogenous phosphorylation patterns under conditions that favor PKG-mediated phosphorylation in homogenates from SCN whose timekeeping mechanisms were free-running in brain slice revealed significant differences in a phosphoprotein of

apparent molecular weight 75 kDa ($p<.05$, ANOVA, Fig. 3). Furthermore, this 75 kDa phosphoprotein displayed significantly more $^{32}PO_4$ incorporation ($p<.05$; Tukey-Kramer) in samples procured at the day and night points at which significantly different levels of endogenous cGMP were measured (CT 11 vs CT 23).

Because monomers of PKG from brain tissue have reported molecular masses of 75-81 kDa and undergo autophosphorylation, we examined the possibility that this PKG substrate showing circadian regulation was itself PKG. The polyclonal antibodies that we raised against the 75 kDa cyclic nucleotide-binding protein extracted from bovine brain bound commercial PKG (Promega Corp.). When an SCN homogenate was incubated with antibody bound to Protein-A/Sepharose beads, the radiolabelled 75 kDa phosphoprotein disappeared from the sample and appeared in the antibody-bead fraction (Fig. 4). This shift could be competitively reduced by preincubation of antiserum with non-radiolabelled commercial PKG. No 75 kDa protein was removed from samples if specific PKG antibodieswere replaced by normal rabbit serum. These results demonstrate that the 75 kDa phosphoprotein is PKG.

Immunoblots of SCN extracts phosphorylated *in vitro* revealed relatively constant levels of PKG between subjective day (CT 10, normalized to 100 percent) and subjective night (CT 23, Fig. 5; normalized 96.8 ± 2.7 percent, $p>.05$), when $^{32}PO_4$ incorporation into PKG is highest and lowest, respectively.

Relative activity of PKG at three time points in the circadian cycle was assessed to determine the ability of the SCN homogenate to phosphorylate the synthetic heptapeptide substrate. Basal activity in exogenously unstimulated samples was significantly higher at CT

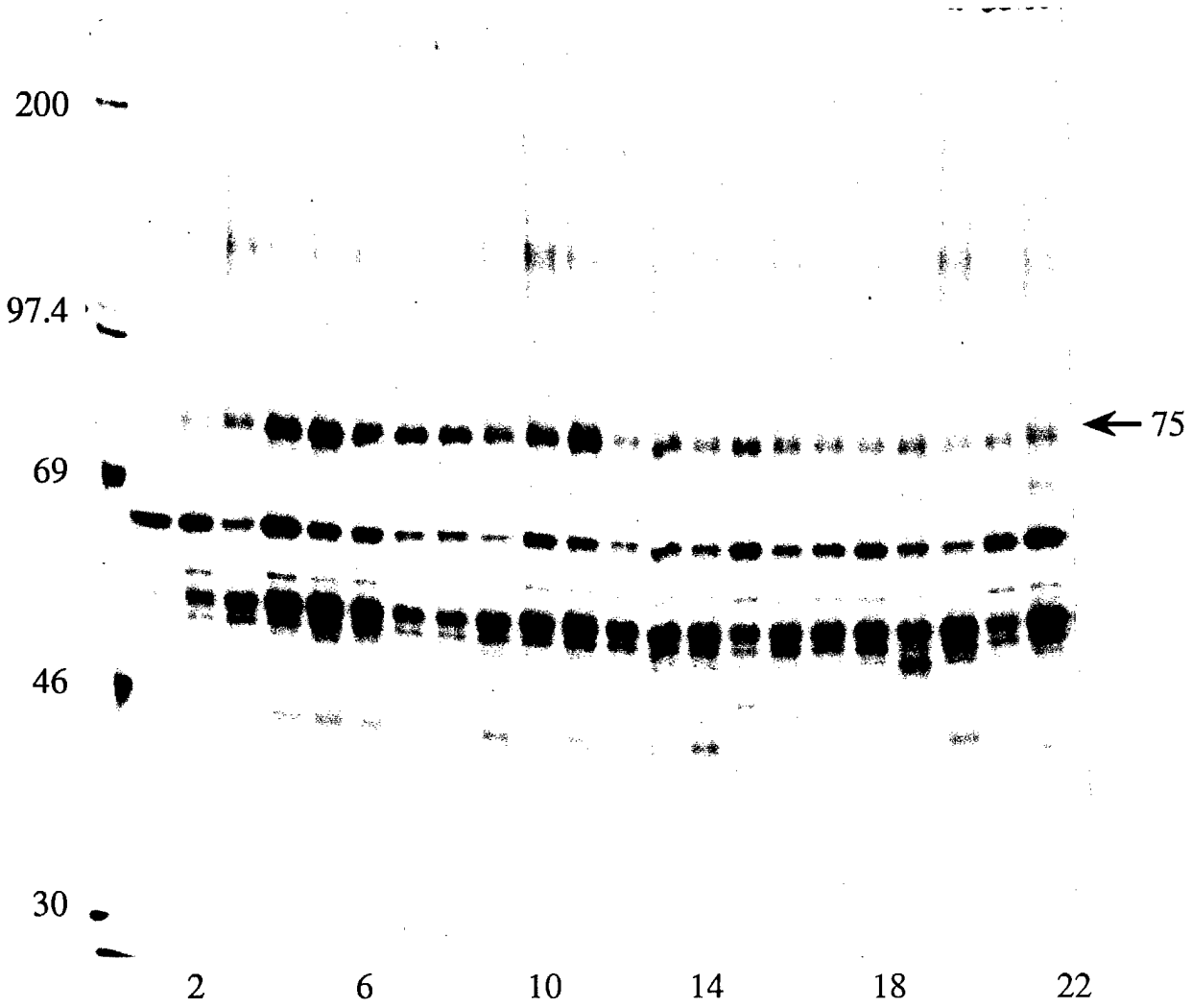


Fig. 3. Autoradiogram demonstrating the circadian pattern of cGMP-stimulated *in vitro* $^{32}PO_4$ incorporation into a 75-kDa phosphoprotein in the SCN. Individual suprachiasmatic nuclei were collected from brain slices at hourly intervals over a 24 hour period, sonicated, subjected to cGMP-stimulated phosphorylation, separated on an 8% gel and analyzed by autoradiography. Time of sampling is indicated at the bottoms of the lanes. CT 16 sample is missing from this gel. Molecular weight markers (kDa) are indicated at the left. The 75-kDa phosphoprotein is indicated at the right.

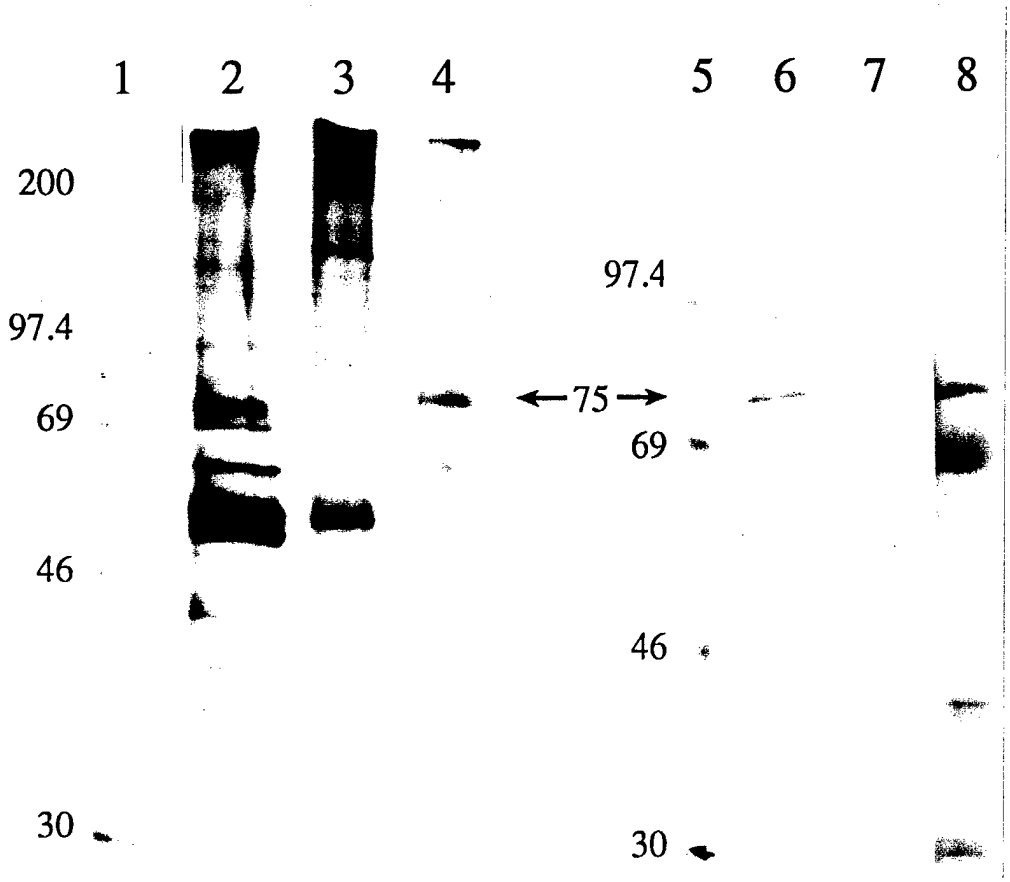


Fig. 4. Polyclonal antibodies which bind the 75-kDa phosphoprotein from the SCN also bind commercial PKG. All samples were subjected to *in vitro* phosphorylation stimulated by cGMP. Autoradiograms were made following SDS-PAGE (15% gels). Control SCN extract is seen in lane 2. The 75-kDa phosphoprotein was precipitated from the SCN homogenate (lane 3) and was released from the PKG antibody/Protein A-conjugated Sepharose bead complex upon addition of Laemmli buffer (lane 4). Control commercial PKG is seen in lane 6. Commercial PKG is extracted from a sample (lane 7) and is released from the antibody complex (lane 8). Molecular weight markers (kDa) are shown for the respective experiments in lanes 1 and 5.

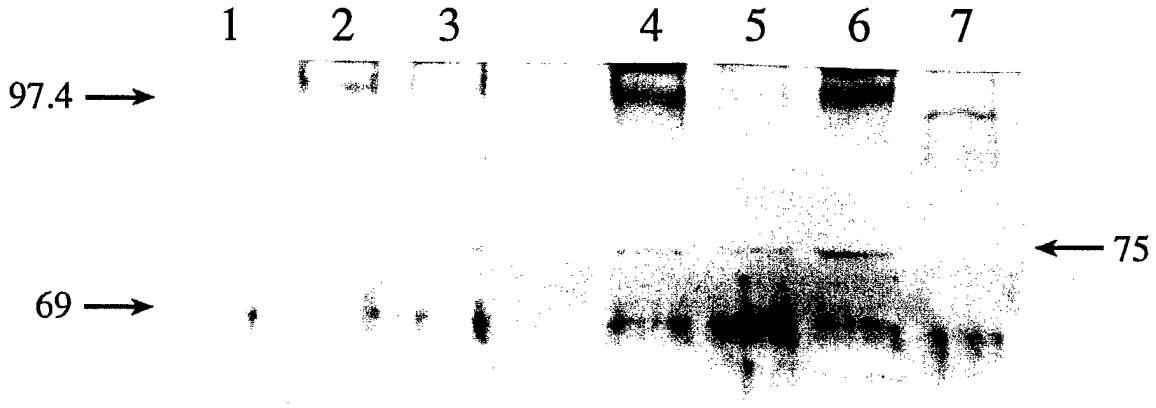


Fig. 5. PKG levels do not change between subjective day and night. Immunoblots of samples of SCN from CT 10 (lanes 2-3) and CT 23 (lanes 4-6), points of high and low $^{32}PO_4$ incorporation, respectively, show relatively constant levels of PKG ($p<.05$). Therefore, protein levels cannot explain the differences in $^{32}PO_4$ uptake between day and night. Molecular weight markers (kDa) are shown in lanes 1 and 7. The molecular mass of native PKG monomers (75 kDa) is indicated at the right.

23 than at CT 7 or CT 18 ($p<.001$), and this increased activity was inhibited by H-89 (Fig. 6). Addition of 20 μM 8-Br-cGMP resulted in approximate 3-4 fold increases in PKG activity (Fig. 6). This maximally stimulated increase in enzymatic activity did not differ over the circadian cycle, suggesting comparable levels of PKG at times when $^{32}PO_4$ incorporation is different.

When the pattern of endogenous cGMP levels was compared to the pattern of $^{32}PO_4$ incorporation into PKG, the levels of cGMP were observed to mirror the degree of $^{32}PO_4$ incorporation over the 24 hour cycle (Fig. 7). Because *in vitro* phosphorylation results in phosphorylation of sites not phosphorylated by normal cellular processes at the time of sampling, changes in $^{32}PO_4$ incorporation into constant levels of PKG must be interpreted as a negative reflection of endogenous phosphorylation state for this protein.

Discussion

The suprachiasmatic nuclei are biological clocks in mammals that possess the ability to generate a 24-hour time base by sequential changes in biochemical processes. In these experiments, it was demonstrated that 1) endogenous activity of PKG is significantly higher at a time in the circadian cycle when both cGMP and autophosphorylation of PKG are highest; 2) endogenous cGMP levels in the SCN vary spontaneously over the circadian period, being highest at the end of the dark portion of the animal's prior 12h:12h light/dark cycle and lowest late in the light portion; 3) the beginning of the endogenous rise in cGMP levels corresponds to the portion of the circadian cycle in which the SCN is most sensitive to phase-shifting by analogs of cGMP; 4) cGMP-dependent protein kinase is present at

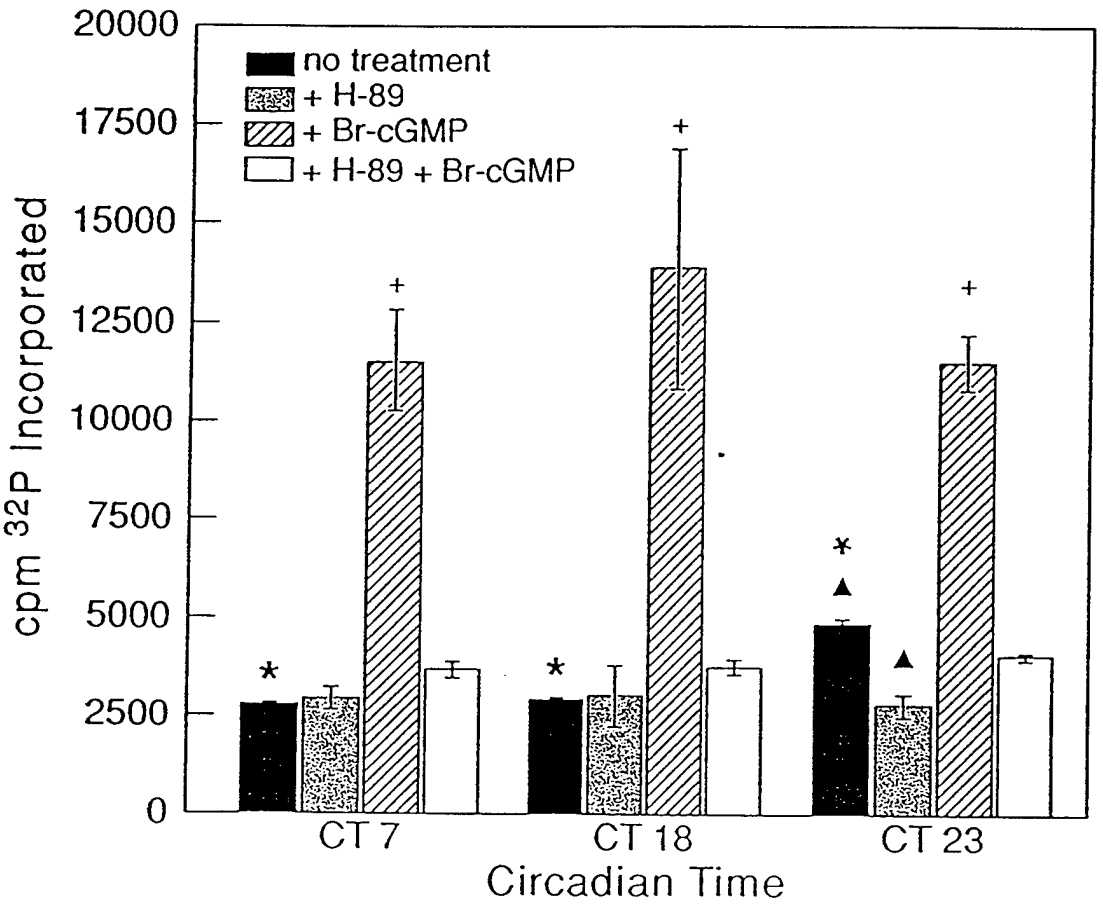


Fig. 6. Endogenous, unstimulated levels of activity of PKG in the SCN (solid bars) are significantly higher at CT 23 than at CT 7 or CT 18 (*; $p<.001$). Stimulatable levels of PKG activity (cross-hatched bars) do not vary over the circadian cycle, but are significantly higher than endogenous activity levels (+; $p<.001$). Inhibition of activity by H-89 under basal (stippled bars; ▲, $p<.01$) and Br-cGMP-stimulated (open bars) conditions in PKA-inhibited reactions demonstrates PKG specificity.

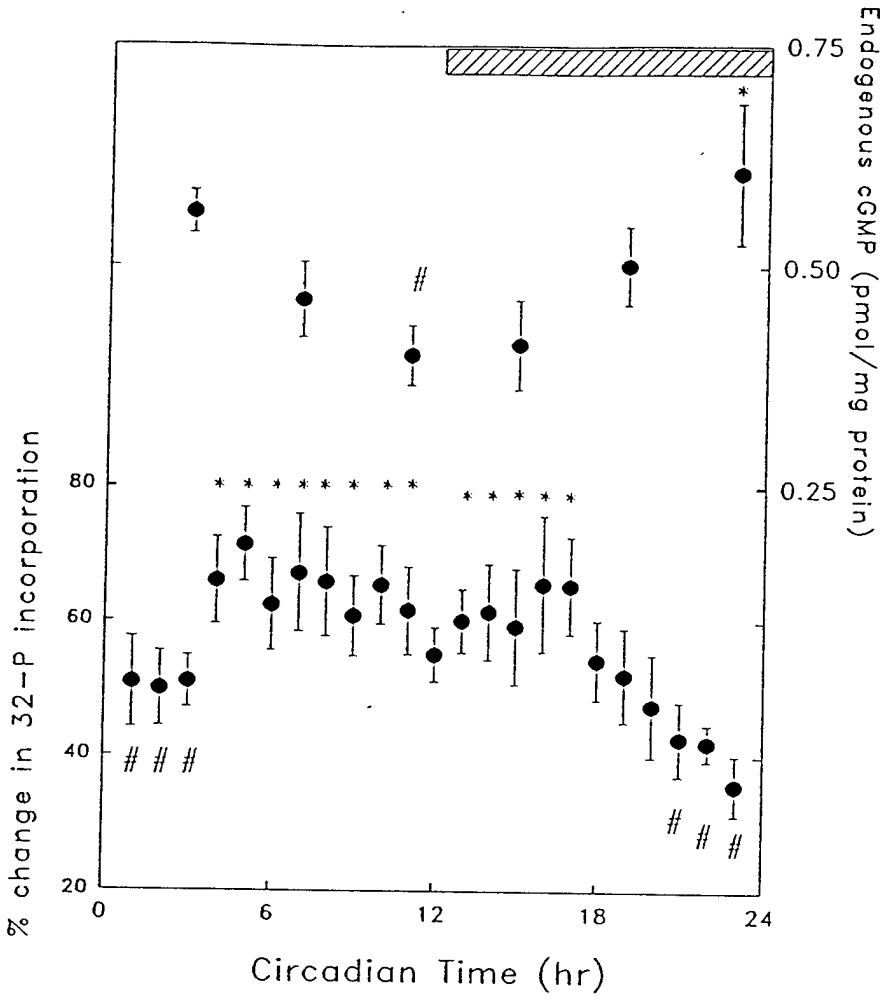


Fig. 7. Levels of endogenous cGMP levels in the SCN are mirrored by cGMP-stimulated $^{32}PO_4$ incorporation into PKG. $^{32}PO_4$ incorporation into PKG (bottom) was quantitated by laser densitometry of autoradiograms and normalized to total $^{32}PO_4$ incorporation per sample, and reflects changes in the endogenous level of phosphorylation of PKG. A significant peak level of cGMP occurs at CT 23 ($p<.05$), a time of significant endogenous phosphorylation ($p<.05$); a significantly low level of cGMP occurs at CT 11, a time of low endogenous phosphorylation of PKG.

relatively constant levels, but incorporates much less $^{32}PO_4$ at times when endogenous cGMP is highest in the SCN, suggesting that the enzyme is already phosphorylated at these times of sampling.

These experiments address the endogenous state of PKG in the unstimulated suprachiasmatic nuclei over the circadian cycle. While changes in phosphorylation state do not prove direct involvement in circadian timekeeping, the significant change in endogenous activity of PKG coincident with the temporally mirrored patterns of endogenous circadian cGMP levels and phosphorylation state of PKG, strongly support that hypothesis. However, the finding that PKG is present and activatable at all times sampled during the circadian cycle suggest that while PKG may be involved in phase-shifting by agents such as 8-Br-cGMP, it is not the temporally restrictive step in that biochemical cascade. This is supported by the observation that analogs of cAMP, which should be capable of activating PKG at high enough concentrations, are ineffective in shifting the phase of circadian rhythms of SCN during subjective nighttime in brain slices (Gillette & Prosser, 1988; Prosser & Gillette, 1989). However, this does not rule out possible roles of PKG and cGMP in circadian timekeeping by the SCN.

In general, phosphorylation of proteins provides a mechanism of regulation which outlasts the direct acute effects of stimuli. Whether the phosphorylation state of PKG is directly related to its enzymatic activity has not been conclusively demonstrated. It is presumed that in these SCN samples, the differences in $^{32}PO_4$ incorporation most likely reflect endogenous phosphorylation state of PKG at the time of sampling. Greater amounts of $^{32}PO_4$ incorporation into SCN samples collected during subjective late day/early night

suggest that more of the enzyme is present in a dephosphorylated state; lower incorporation suggests that in samples from late subjective nighttime, the enzyme may be autophosphorylated, rendering it less able to incorporate $^{32}PO_4$ in the subsequent *in vitro* reaction. Functionally, the phosphorylation state of PKG is the most likely explanation for the changes in activity of PKG demonstrated in unstimulated SCN. Phosphorylation state could also regulate localization of the enzyme within the cell, or changes in substrate binding affinity.

While there is significant day-night variation in measured cGMP levels, the amplitude of the change in the entire SCN varies less than one order of magnitude over the circadian period. The SCN is a heterogenous population of cells by a number of measures, including peptide content, neuronal activities, and response characteristics (van den Pol, 1980; Gillette *et al.* 1995). If the circadian fluctuation in cGMP levels occurs only in a subset of the population of cells, the circadian differences could be obscured by levels of cGMP in non-oscillatory neighboring cells. This possibility should be resolved with methods other than RIA, but this is technically difficult due to the relatively small amount of tissue that is the SCN. It is likely, however, that the different affinities of the two cGMP-binding sites per subunit of PKG and the positive cooperativity of binding impart a heightened sensitivity of the enzyme to subtle fluctuations in basal cGMP levels through the interaction of the two sites. It is also possible that localization or compartmentalization of PKG and/or cGMP within the suprachiasmatic nuclei or within the neurons of this brain region may create much greater concentration differences than these small but significant differences measurements *in vitro*.

Cellular constituents such as cGMP and PKG may be involved in a range of other functions in the SCN which are not directly related to circadian timekeeping. It follows that the circadian changes in cGMP levels may be occurring only in a subset of cells of the SCN. This raises the possibility that cyclic changes in cGMP are occurring in a subset of cells involved in SCN output rather than timekeeping. However, the discrete period of phase-shifting effectiveness of cGMP analogs (Prosser *et al.* 1989) closely precedes the endogenous increases in basal levels of cGMP that we report here. This temporal correlation between period of action by an analog of cGMP on the pacemaker mechanism and pacemaker-regulated increases in cGMP levels suggests that these two states may be functionally related, rather than operating as separate input-output elements.

One may speculate that the cellular machinery responsive to cGMP is "ready and waiting" for the endogenous increase in cGMP level. Stimulation with a cyclic nucleotide analog prior to the endogenous rise in cGMP levels may advance the time-keeping mechanism to a specific temporal biochemical state. This suggests that, in addition to a role in timekeeping, cGMP and PKG may play roles in signal transduction to the SCN. This is supported by the observation that the SCN *in vivo* are sensitive to phase-shifting by light stimuli only during subjective night, and neurotransmitters such as glutamate (Ding *et al.* 1994) and acetylcholine (Liu & Gillette, 1994) that work through cGMP-mediated signal transduction mechanisms are also most effective several hours prior to the peak in endogenous cGMP. Thus, "nighttime" stimuli such as light pulses or cGMP analogs may be prematurely stimulating a PKG pathway which would normally respond to the endogenous rise in cGMP level several hours later. As well, such stimuli may produce even greater

increases in cGMP than are seen endogenously, under unstimulated conditions, producing more dramatic biochemical effects than those seen under unstimulated conditions.

In conclusion, we have shown that PKG in the suprachiasmatic nuclei of the rat shows variation in activity and in $^{32}PO_4$ incorporation with a near 24-hour period, while enzyme level remains relatively constant. In addition, activity levels and the pattern of $^{32}PO_4$ incorporation mirror the endogenous levels of cGMP, which also vary significantly over the circadian cycle. Both cGMP and intrinsic PKG phosphorylation peak approximately 5 hours after the peak sensitivity of the SCN to phase-resetting by cGMP analogs. These temporal relationships suggest that the cGMP and PKG pathways interact with the circadian timekeeping system daily and may underlie the biochemical transition from the clock's night to day.

Chapter 3. NEUROCHEMICAL ANALYSIS OF SIGNAL TRANSDUCTION MECHANISMS IN THE PHOTIC ENTRAINMENT PATHWAY TO THE SUPRACHIASMATIC NUCLEUS.

Experiments described in this section were conducted in the laboratory of M.A. Rea, Biological Rhythms and Integrative Neuroscience Center, Armstrong Laboratory (CFTO), Brooks AFB, TX, in conjunction with the author's Air Force Graduate Research Fellowship, 1991-1994.

A portion of this data is presently in press (Ding *et al.* 1994).

Introduction

The light-entrainable circadian oscillator responsible for the generation of circadian rhythms in mammals has been localized to the suprachiasmatic nucleus (SCN) (Rusak & Zucker, 1979; Meijer & Rietveld, 1989). SCN-driven circadian rhythms are entrained to the environmental light-dark (LD) cycle as a consequence of daily, light-induced adjustments in the phase of the circadian oscillator. Light at dusk causes phase delays, while light exposure at dawn results in phase advances of the circadian oscillator (DeCoursey, 1960; DeCoursey, 1964; Daan & Pittendrigh, 1976). Light exposure at circadian phases that result in phase shifts of the circadian oscillator induce expression of a number of immediate early genes (IEG)(Rea, 1989a; Aronin *et al.* 1990; Kornhauser *et al.* 1990; Earnest *et al.* 1990; Rusak *et al.* 1990; Kornhauser *et al.* 1992), including *c-fos*, among discrete populations of SCN cells. Because light-induced expression of the *c-fos* product, Fos, in the SCN is proportional to the light intensity and shows similar temporal restrictions as phase-shifting, Fos has been postulated to play a role in photic entrainment (Rea, 1989a; Aronin *et al.* 1990; Kornhauser *et al.* 1990; Earnest *et al.* 1990; Rusak *et al.* 1990; Kornhauser *et al.* 1992; Rea *et al.* 1993b).

Potentially entraining photic information is conveyed to the SCN principally via a monosynaptic projection from retinal ganglion cells (Moore & Lenn, 1972; Pickard, 1982; Johnson *et al.* 1988b), the retinohypothalamic tract (RHT). Considerable evidence suggests that RHT neurotransmission is mediated by the release of an excitatory amino acid (EAA) from RHT terminals (Liou *et al.* 1986; Kim & Dudek, 1991; deVries *et al.* 1993; Abe *et al.*

1991; Abe *et al.* 1992; Colwell & Menaker, 1992; Colwell *et al.* 1991; Colwell *et al.* 1990; Rea *et al.* 1993a). The intercellular messenger, nitric oxide (NO), has been implicated in EAA signal transduction in the brain (Garthwaite *et al.* 1988; Bredt & Snyder, 1989). A previous study demonstrated that light-induced increases in heart rate are attenuated by inhibitors of NO synthase microinjected near the rat SCN (Amir, 1992), raising the possibility that this response to photic stimulation may involve NO production in the region of the SCN. Furthermore, Ding *et al.* demonstrate that EAA agonists induce phase shifts of the circadian rhythm in firing rate of SCN neurons in a brain slice preparation, and that this occurs through an NO-dependent mechanism (Ding *et al.* 1994). In order to determine whether NO is involved in the transduction of photic information to the SCN circadian oscillator, we examined the effects of local administration of the competitive NOS inhibitor, L-nitroarginine methyl ester (L-NAME), on light-induced phase shifts of the free-running activity rhythm and on light-induced *c-fos* expression in the hamster SCN (Section A).

The SCN in brain slices has been shown to be differentially sensitive to cyclic nucleotide analogs, depending on time of application during the circadian cycle (Gillette & Prosser, 1988; Prosser & Gillette, 1989; Prosser *et al.* 1989). Analogs of cGMP advance the phase of the rhythm only during subjective night. The coincidental overlap of temporal sensitivities of the SCN to light and cGMP suggest a possible mechanism by which effects of light input are conveyed into cellular signals involved in SCN entrainment. The putative neurotransmitter for the optic projections to the SCN is glutamate (Liou *et al.* 1986; Cahill & Menaker, 1987; Cahill & Menaker, 1989b), and NMDA-type receptors have been implicated in RHT responses (Ohi *et al.* 1991; Kim & Dudek, 1991). Glutamate has been

associated with increases in cGMP in other brain areas, many of which are transduced via a nitric oxide mechanism (Bredt & Snyder, 1989; Danysz *et al.* 1989; Ferrendelli *et al.* 1974; Garthwaite *et al.* 1988).

If the pathway leading to light-induced phase shifts works through stimulating guanylate cyclase, the cGMP levels should rise after photic stimulation. Cyclic nucleotides are heat stable molecules, which makes them suitable for measurements or cellular localization following microwave fixation. This method of tissue fixation has the advantages of being quite rapid compared to decapitation, dissection and freezing or acid treatment of brain tissue, which often allows degradation of cyclic nucleotides (Barsony & Marx, 1990), and of preserving the structure of the cyclic nucleotide being measured, which is often altered by chemical fixation techniques (de Vente *et al.* 1989). Microwave fixation of cultured tissue halts enzymatic activity while having no detrimental effects on the heat-stable cyclic nucleotides (Barsony & Marx, 1990). Therefore, we undertook measurements of cGMP levels in SCN microwave-fixed *in vivo* following photic stimulation, in order to test the hypothesis that light signals to the SCN are transduced via a cGMP pathway (Section B).

Section A: Nitric oxide is necessary for photic phase-shifting.

Methods

Syrian hamsters (*Mesocricetus auratus*) weighing 100 - 110 g were obtained from Charles River (Wilmington, MA) and housed under a LD 14:10 lighting cycle for at least 2 weeks prior to surgery. Hamsters received cannula guides stereotaxically aimed at the floor of the third ventricle near the SCN. Under pentobarbital anesthesia (90 mg/kg, i.p.), 26g cannula guides were stereotaxically implanted to a depth of 2.9 mm below the dura and fixed to the skull with fine machine screws and dental cement. Cannula guides were fixed at stereotaxic coordinates of 1.0 mm anterior to bregma at the midline (upper incisor bar at 0). Animals were allowed to recover for 1 week under LD 14:10 (Rea *et al.* 1993a). After recovery from surgery, animals were transferred to individual cages with 9 inch running wheels and maintained in constant darkness (DD). Wheel running activity was monitored continuously on a computer running Dataquest III data acquisition software (Minimitter Co., Sunriver, OR). The onset of wheel running activity, designated as circadian time (CT) 12, was used as a phase reference point for the timing of photic stimulation. The period of the free running rhythm was calculated as the average amount of time between activity onsets over the five days prior to stimulation. The onset of activity on the day of stimulation was predicted by extrapolation of the regression line fitted to activity onsets from the five days preceding the day of stimulation. After stable free-running activity rhythms were established (10-14 days), animals received i.c.v. injections of either (a) artificial cerebrospinal fluid (ACSF; 122 mM NaCl, 3.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4,

25 mM $NaHCO_3$, and 1.2 mM $CaCl_2$), (b) 0.1 mM N-nitro-L-arginine methyl ester (L-NAME) in ACSF, (c) 1 mM L-NAME in ACSF, (d) 1 mM L-NAME plus 4 mM arginine in ACSF, or (e) 1 mM D-NAME. Most of these animals received light stimulation (20 lux white light for 5 minutes) beginning 10 minutes after injection. Treatments were administered at either 2 circadian hours (1 circadian hour= free-running period/24) after the predicted activity onset (CT 14), or 7 circadian hours after the predicted activity onset (CT 19). After photic stimulation, the hamsters were returned to darkness and wheel running activity was monitored for an additional 10-14 days. Phase shifts were calculated as the differences between the estimates of CT 12 on the day after stimulation as determined by (a) back extrapolation of the regression line fitted to activity onsets on days 4 through 9 after stimulation, and (b) extrapolation of the regression line calculated from activity onset data collected during the five days prior to and including the day of stimulation (Rea *et al.* 1993a).

In order to further elucidate the mechanism by which L-NAME-sensitive photic information is transduced in the SCN, the effects of L-NAME on photically-stimulated Fos production were examined. Cannulated hamsters were maintained under DD in cages equipped with running wheels. Animals received injections of either ACSF, L-NAME, or L-NAME plus arginine, as described previously herein, at CT 19, a time in the circadian cycle at which light induces both Fos expression and phase advances of the activity rhythm (DeCoursey, 1964; Daan & Pittendrigh, 1976; Rea, 1989a; Aronin *et al.* 1990; Kornhauser *et al.* 1990; Earnest *et al.* 1990; Rusak *et al.* 1990; Kornhauser *et al.* 1992). After light stimulation, hamsters were returned to darkness. Hamsters were deeply anesthetized 90

minutes after the onset of the light stimulation and perfused transcardially with 100 ml of heparinized phosphate buffered saline (pH 7.4), followed by 100 ml of 4% paraformaldehyde in sodium phosphate buffered saline (pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde overnight at 4°C, followed by 24 hours at 4°C in 0.1 M sodium phosphate buffer (pH 7.4). Seventy micron-thick frontal sections were cut on a vibratome and incubated overnight at 4°C in Fos antiserum (c-fos Ab-2 @ 1:2000; Oncogene Science, Manhasset, MY). This antiserum was raised against a synthetic peptide (SGFNADYEASSSRC) corresponding to residues 4-17 of human Fos. Fos-like immunoreactivity (Fos-lir) was detected using a Vectastain ABC kit (Vector Labs, Burlingame, CA).

Results

Hamsters that received photic stimulation after injection of ACSF showed stable phase advances of the free-running activity rhythm of 89 ± 7 minutes (mean ±SEM; n=7), while similar treatment at CT 14 resulted in stable phase delays of -71 ±20 minutes (n=4)(Figs. 8, 9). Administration of 1 mM L-NAME prior to light exposure markedly attenuated light-induced phase advances and delays (Figs. 8, 9). This effect was reversible and dose-dependent (Fig. 10). Furthermore, the effect of 1 mM L-NAME was blocked by co-injection of 4 mM arginine (Fig. 10), which overcomes the competitive blockade of nitric oxide synthase by the inhibitor. D-NAME did not significantly attenuate photic phase shifts (Fig. 8).

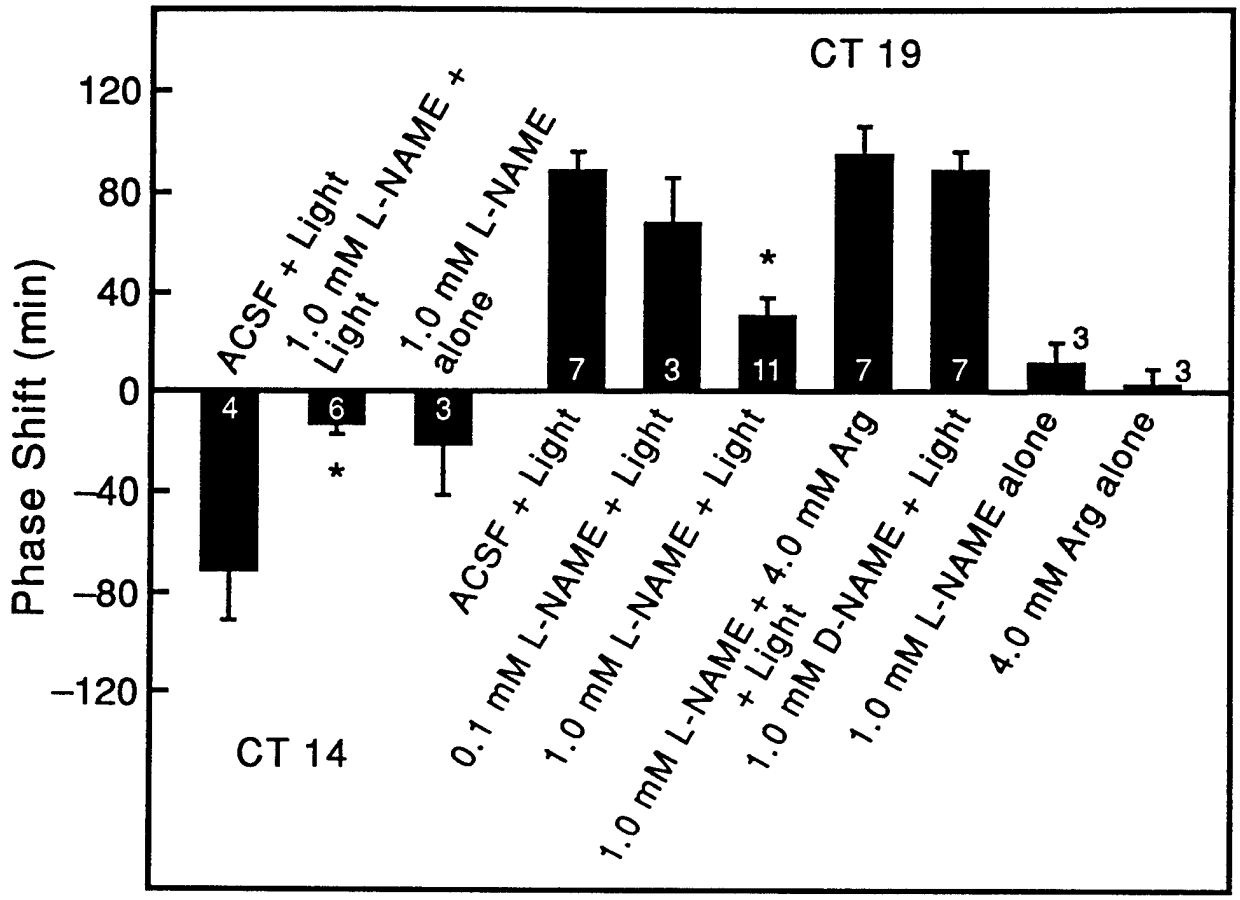


Fig. 8. L-NAME inhibits photic phase shifts, in a dose-related manner. Data represent the mean ± SEM of the number of determinations indicated in each bar. Asterisks (*) indicate statistically significant differences relative to the vehicle + light group.

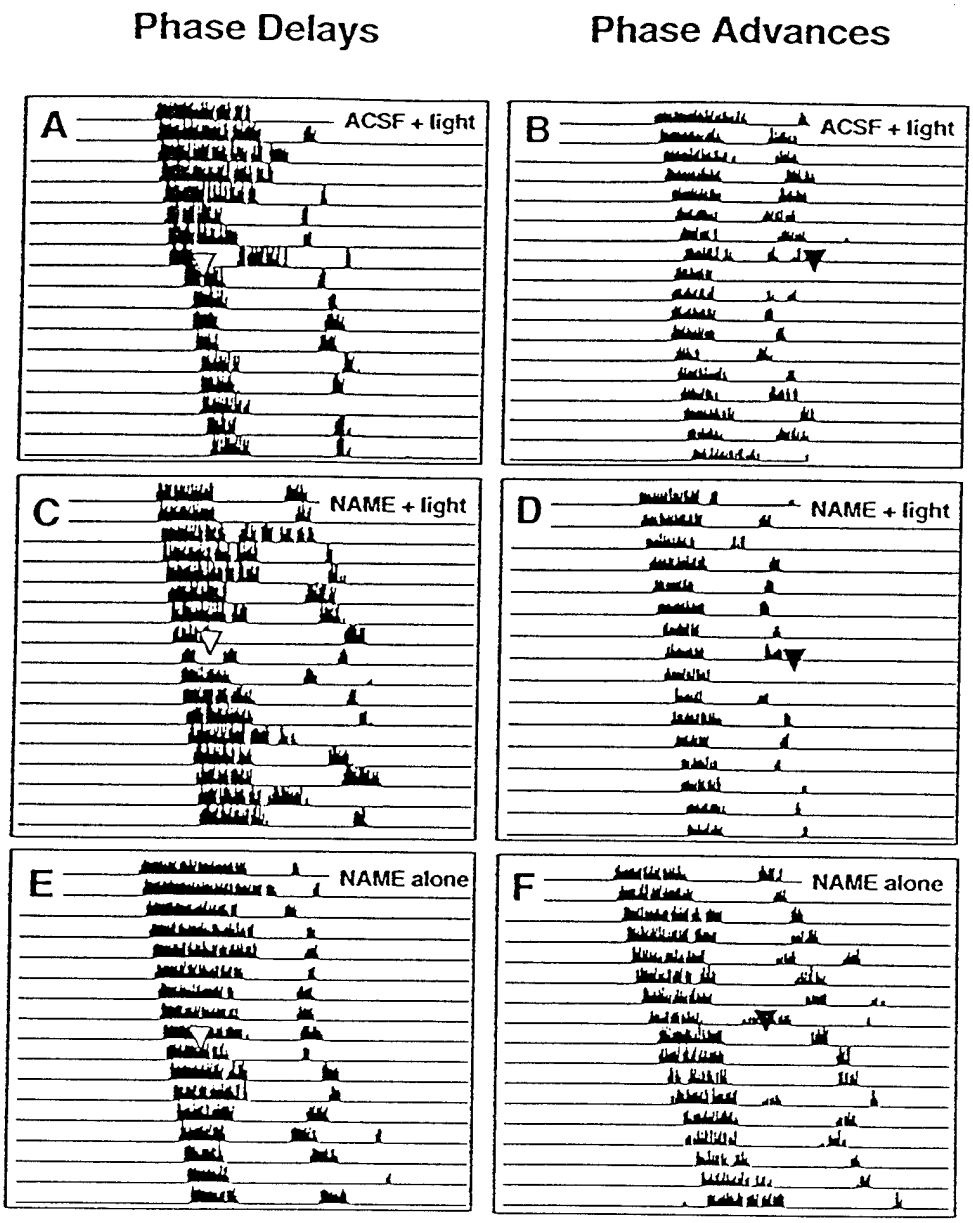


Fig. 9. Intracerebroventricular administration of L-NAME inhibits light-induced phase shifts of the free-running activity rhythm in hamsters. Shown are representative actograms of individual hamsters that received the following treatments at the times indicated by the inverted triangles. A,B: icv injections of 0.5 μl ACSF followed by exposure to 20 lux of white light for 5 minutes. C,D: icv injections of 0.5 μl of 1 mM L-NAME in ACSF followed by exposure to 20 lux of white light for 5 minutes. E,F: icv injections of 0.5 μl of 1 mM L-NAME in ACSF without light exposure. Light stimulation was initiated at either CT 14 (▽: left panels) or CT 19 (▼: right panels). Horizontal lines represent successive days while vertical bars indicate 6 minute bins during which wheel running activity occurred. The height of the vertical bars is proportional to the number of wheel turns that occurred during that 6 minute period.

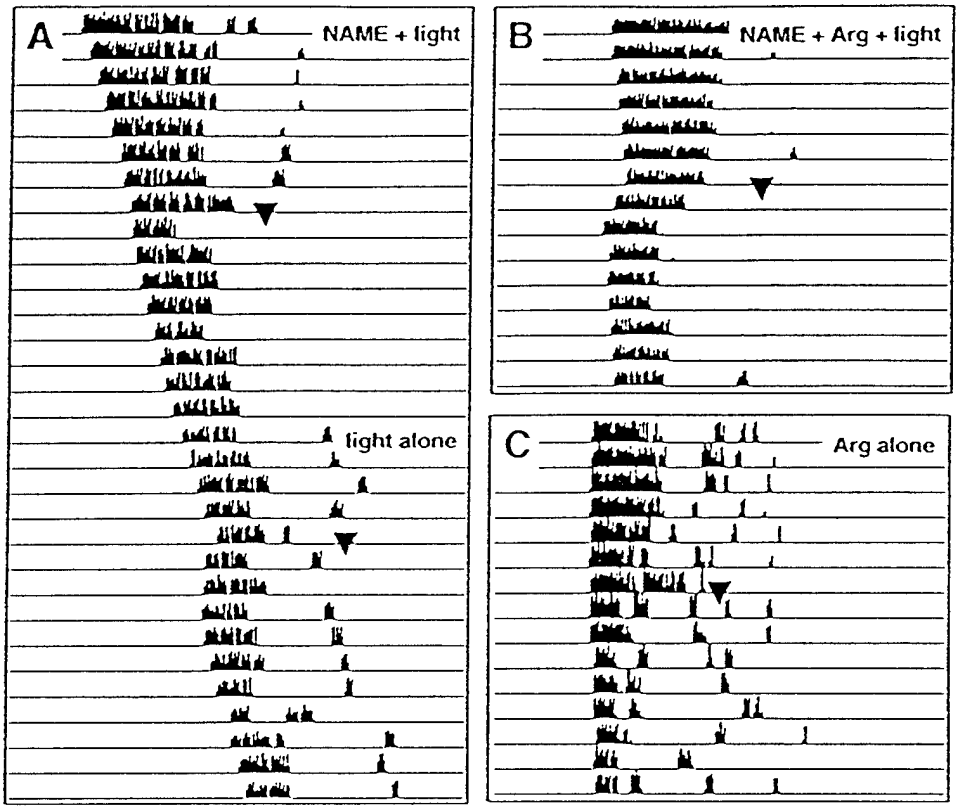


Fig. 10. The effect of L-NAME injection is reversible and blocked by co-administration of arginine. Shown are representative actograms of individual hamsters that received the following treatments at the times indicated by the inverted triangles (▾). A: Reversibility. Hamster received icv injection of 1 mM L-NAME followed by exposure to 20 lux of white light for 5 minutes at CT 19. Thirteen days later the same hamster received an additional light exposure at CT 19 without injection of L-NAME. B: Hamster received icv injection of 1 mM L-NAME + 4 mM arginine in ACSF followed by exposure to 20 lux of white light for 5 minutes at CT 19. C: Hamster received icv injection of 4 mM arginine in ACSF at CT 19 without light exposure.

Neither 1 mM L-NAME (Fig. 9) nor 4 mM arginine (Fig. 10) administered alone altered the phase of the circadian activity rhythm (Fig. 8). These results suggest that the transduction of photic entrainment signals in the SCN requires nitric oxide formation.

Light stimulation of animals receiving ACSF resulted in characteristic patterns of Fos expression in the SCN. In three independent experiments, pretreatment with L-NAME did not appear to alter the pattern of light-induced Fos immunoreactivity in the SCN (Fig. 11).

Discussion

The results of this study, in conjunction with the findings of Ding *et al.* (Ding *et al.* 1994), provide strong evidence that photic phase shifts of the SCN circadian oscillator require NO production, while light-induced Fos expression does not.

These results, and others (Rea *et al.* 1993b), argue that *c-fos* expression is not sufficient to mediate photic phase shifts, although a role for Fos in this process cannot be eliminated. However, based on our results, Fos production is not dependent on nitric oxide production, and thus suggests that either parallel pathways exist which mediate transcription of this IEG, or that Fos production is upstream of NO synthesis. In order for the latter consideration to fit with our data, the L-NAME injected into the region of the SCN would have to inhibit NOS sufficiently long after the time required for Fos production, typically 90-120 minutes (Rea, 1989a). This hypothesis can easily be tested by injecting L-NAME *after* photic stimulation has resulted in production of *c-fos* mRNA.

More likely, there are parallel pathways which regulate the production of Fos, which are also activated by photic stimuli. Indeed, a recent study demonstrated that i.c.v. injection

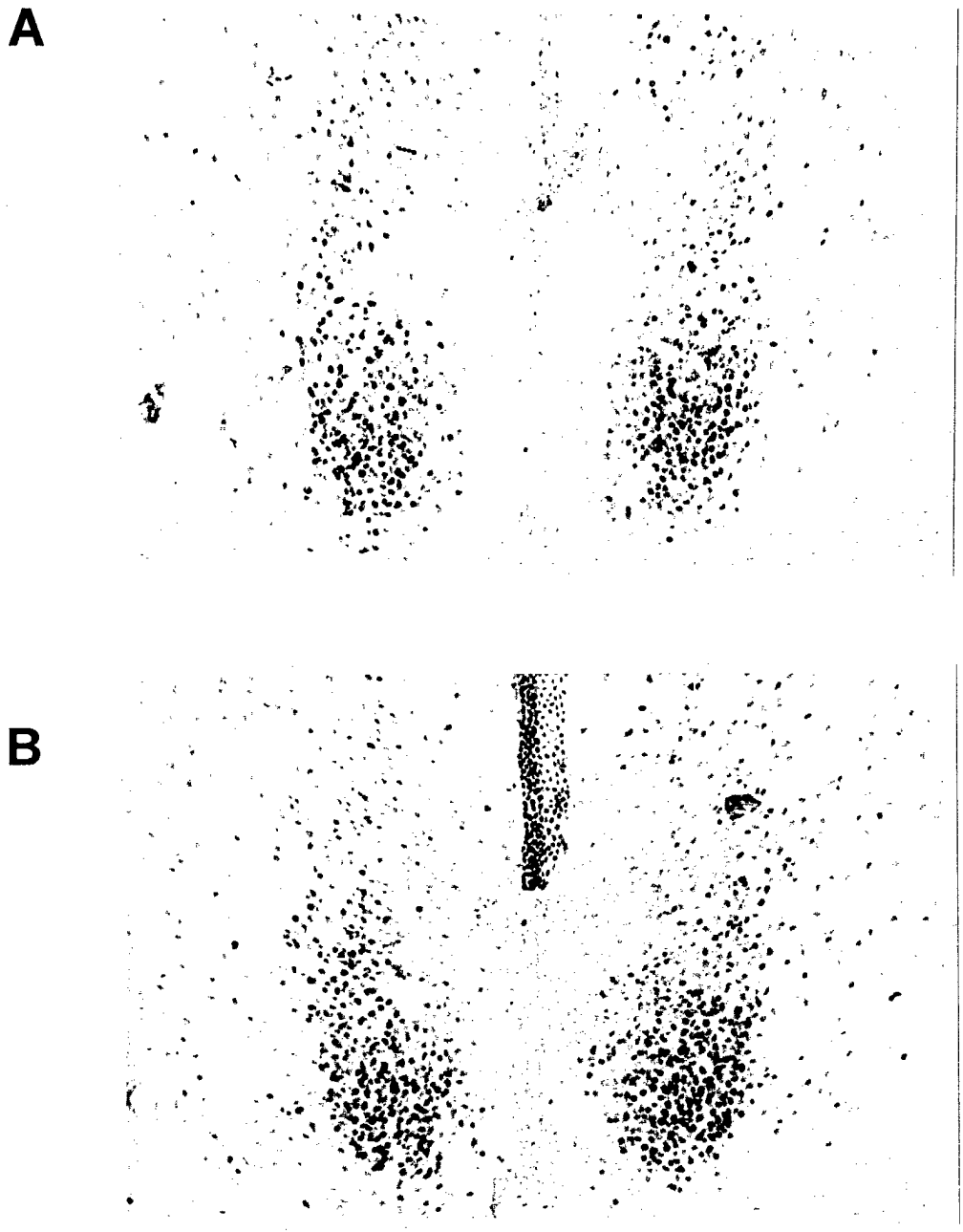


Fig. 11. Intracerebroventricular administration of L-NAME does not inhibit light-induced Fos expression. Shown are representative photomicrographs of individual hamsters that received icv injections of either (A) ACSF or (B) 1 mM L-NAME 10 minutes prior to exposure to 20 lux of white light for 10 minutes at CT 19. The number of Fos-immunoreactive cell nuclei observed in the SCN after photic stimulation following icv injection of ACSF (730 ±140/SCN; n=4) or 1 mM L-NAME (660 ±160/SCN; n=3) did not differ significantly (Students' two-tailed "t" test; $p > 0.5$).

of KN-62, a calmodulin kinase inhibitor, significantly attenuates light-induced phase shifts (Golombek & Ralph, 1994). Whether these pathways act in conjunction with NO pathwaysto produce photic phase-shifts or have no effect on photic phase-shifting has yet to be determined.

Finally, since NO appears to act primarily, if not exclusively, as a transcellular messenger (Garthwaite, 1991; Southam & Garthwaite, 1991; Ding *et al.* 1994), photic information may be conveyed to the SCN circadian oscillator by a nonsynaptic mechanism, raising the possibility that both neurons and neuroglia may participate in photic regulation of circadian rhythmicity.

Section B: Measurement of cGMP after photic stimulation in vivo

To further investigate the mechanisms involved in photic entrainment of SCN-generated circadian rhythms, we attempted to examine the effects of light on endogenous levels of cGMP in the SCN. Experiments were designed to measure cGMP and cAMP levels by radioimmunoassay in SCN from brains microwave-fixed *in vivo* following photic stimulation at a (circadian) time and intensity sufficient to evoke phase shifts in circadian locomotor rhythms.

Methods

Light phase shifts

Male Syrian hamsters (75-100 g) were maintained on a 14:10 light/dark cycle for >2 weeks prior to experiments. Hamsters were used in this set of experiments due to the

consistency of their running wheel activity patterns. At the desired time of the circadian cycle, animals were placed individually in a lighting box and subjected to 3000-3250 lux of full-spectrum white light for 30 seconds. Controls were placed in the lighting box without light. Animals were then placed in constant darkness and their locomotor rhythms were monitored for 8-12 days. Shifts in onset of activity rhythms were analyzed by methods previously described (Rea, 1993a).

Cyclic nucleotide measurements

At CT 19, a time of the circadian cycle at which photic stimulation evokes advances in circadian locomotor rhythms, hamsters were placed individually in a tubular plastic holder with their incisors over a bite bar. The holder was constructed so as to position the head of the animal in the microwave guide (Dr. P. Werchan, Brooks AFB).

Animals were subjected to 3000-3250 lux of white light for either 30 seconds or 5 minutes, or were subjected to similar periods in the microwave holder without light, as controls. Brains were then fixed by 750 mseconds of 3 kW microwave radiation focused on the head. For RIA #1 and #2 brains were removed, frozen in plastic scintillation vials on dry ice, then placed in -20°C overnight. For RIA #3, brains were removed following microwave fixation and were immediately sliced, frozen and the SCN removed. For comparison, brains from freshly decapitated animals were sampled in similar fashion. Suprachiasmatic nuclei were removed by making an approximate 500 μm coronal slice of the brain containing the optic chiasm, placing the slice on a frozen microscope slide and punching out the paired nuclei with a frozen modified 18 g needle (I.D.= 840 μm)(Palkovits, 1973). SCN punches were either stored singly or pooled, in Eppendorf tubes at -80°C.

Microwave fixation efficiency

Efficiency of microwave fixation of the brain was measured by its effects on phosphodiesterase (PDE) activity. Samples from hypothalamus, cortex, and cerebellum were assayed. Single tissue punches were sonicated in 120 μl of 40mM Tris-HCl, pH 8.0, with 5 mM 2-β-mercaptoethanol, and centrifuged 5 minutes at 10,000 g at 4°C. Phosphodiesterase activity assays, in which degradation of an exogenous mixture of cGMP and [^{3}H]-cGMP by endogenous PDE is measured, were performed according to the batch procedure of Thompson and Appleman (1971) with the addition of 0.1 mM $CaCl_2$ to the reaction mix (Sharma *et al.* 1984). Final reaction mixture contained 100 µl tissue supernatant, 40 mM Tris-HCl, 5mM 2-β-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, approximately 200,000 cpm [^{3}H]-cGMP (~45 pM), and 200 μM cGMP in a total volume of 400 µl. Primary reactions were carried out at 30°C for 20 min, stopped by boiling 45 seconds and placing on ice. Secondary reactions, in which [^{3}H]-GMP generated by the PDE reaction is further degraded to (uncharged) [^{3}H]-guanosine, were carried out with addition of 100 µl cobra venom (1 mg/ml; SIGMA), 10 min, 30°C. Separation of [^{3}H]-guanosine from unreacted tracer was accomplished with addition of BioRad AG1-X2 anion exchange resin (1 ml, 20% methanol slurry) and equilibration for 15 minutes at 4°C with occasional remixing. Samples were centrifuged 5 minutes at 10,000*g* and 500 µl supernatant removed for liquid scintillation counting in 10 ml Optifluor scintillation cocktail. Calcium-sensitivity of the reaction was tested for with additions of 0 to 10 mM Ca^{2+} to samples aliquots from a hypothalamus extract from 2 freshly dissected brains.

Cyclic nucleotide measurement

Tissue samples were sonicated in 400 μl 6% trichloroacetic acid, 4° C and centrifuged 5 minutes at 10,000*g*. Supernatant was removed and the TCA neutralized with addition of $CaCO_3$ (Tihon *et al.* 1977). Aliquots of supernatant were removed for cGMP RIA (100 μl) and cAMP RIA (50 μl). Equal volumes of 2X sodium acetate buffer (0.5M, pH 6.2) were added to allow acetylation of samples prior to assay by addition of 5 μl of acetic anhydride/triethylamine (1:2). Samples were assayed for cGMP and cAMP contents by commercially available radioimmunoassay kits (New England Nuclear). Cyclic nucleotide contents of samples were calculated using a log-logit transformation software program. Cyclic nucleotide levels were analyzed both "per pellet" and "per protein content". Protein content was measured by Bradford assay (Bradford, 1976), using BSA as a standard.

Results

Phosphodiesterase assays

Microwave fixation successfully halted all PDE activity in SCN (n=13), as well as hypothalamic (n=10) and cortical (n=4) samples, compared to activity measured from freshly dissected SCN (57.7 ± 8.7 nmol cGMP/30 min/punch, n=5). Single 18 g punches contain approximately 30-50 μg protein. Reactions were independent of Ca^{++} added in the reaction buffer up to 10 mM (1.90 ±0.15 nmol cGMP/30 min/aliquot).

Cyclic nucleotide radioimmunoassays

Radioimmunoassays performed on SCN from microwave-fixed hamster brains, regardless of light treatment, yielded highly variable measurements (Table 2). Cyclic

nucleotide levels normalized "per punch" were as highly variable as those normalized to measured protein content, indicating that the variability is not due, at least entirely, to protein assay variability.

Table 2. Levels of cGMP and cAMP in SCN of microwave-fixed hamster brains at circadian time 18, normalized both to sample size (per tissue punch) and to sample protein content (per mg protein). Hamsters were immobilized in holders and subjected to light for either 30 seconds or 5 minutes. Control animals were immobilized for similar periods of time, under dim red light. For comparison, SCN were dissected from hamsters following decapitation and immediately frozen, at CT 18 under dim red light.

	cGMP levels		cAMP levels		
	pmol/punch	pmol/mg	pmol/punch	pmol/mg	n
control (30 sec)	.092±.104	6.80±3.64	.358±.100	25.6±15.4	7
light (30 sec)	.099±.018	7.32±2.87	.406±.202	32.6±24.8	7
control (5 min)	.082±.007	6.28±4.56	.346±.180	21.7±11.2	7
light (5 min)	.092±.015	6.06±3.38	.408±.235	26.0±24.3	7
fresh dissect	.078±.011	4.42±0.95	.717±.299	40.6±21.2	6

Discussion

Microwave fixation of hamster brains *in vivo* successfully halted phosphodiesterase activity in SCN, as well as lateral hypothalamus and cortex, samples. The addition of calcium to the reaction procedure was performed to ensure that activity of any calcium-dependent cGMP PDEs would be accounted for. The observation that there was no difference in PDE activity in the hypothalamic extracts with addition of Ca^{2+} implies that there was sufficient calcium in the extracts to activate such PDEs, or that there was little or no calcium-dependent cGMP PDE present in the extracts.

As can be seen in Table 2, cyclic nucleotide measurements from the microwaved brains were highly variable. This may be due to several procedural variables. Protein content as measured by the Bradford assay was not extremely reliable due to the difficulty in obtaining a resuspendable pellet from microwave-fixed tissue. On the other hand, making slices of frozen brains from which to take SCN punches, either by using razor blades in the slotted brain mold or by hand with a razor blade, yield slices of highly variable thicknesses, so that resulting SCN punch sizes cannot be assumed similar, and is thus not an accurate unit to which measurements can be normalized. Thus, either of the normalization techniques (per punch or per protein) may have been the cause for the high level of variation among samples.

Despite the sample variability, sample means were fairly unchanged between controls and treated groups. While cyclic nucleotide levels did not appear to change dramatically following light stimulation, it may be that cyclic nucleotide levels are altered only in a subset of cells in the SCN, and that this difference may be obscured by the cyclic nucleotide content of the remaining cells in the sample. Such a localized effect would not be unusual given the demonstrated patterns of Fos staining (Rea, 1989a) following light treatments.

While this method may not have been successful in demonstrating localized effects of light on cyclic nucleotide levels, we did demonstrate that the method of microwave fixation following treatments of the SCN has been shown to rapidly fix brain tissue sufficiently to stop phosphodiesterase, and, most likely, all enzymatic activity, and may prove to be a valuable tool for future experiments requiring sampling within short durations following phase-shifting stimuli.

CHAPTER 4. PUTATIVE REGULATION OF cAMP-RESPONSE ELEMENT BINDING PROTEIN (CREB) BY PKG IN THE LIGHT ENTRAINMENT PATHWAY TO THE SUPRACHIASMATIC NUCLEI.

Introduction

It is established that the suprachiasmatic nuclei (SCN) generate and regulate circadian rhythms in mammals (Rusak & Zucker, 1979; Meijer & Rietveld, 1989). However, the molecular mechanisms underlying synchronization of SCN-generated rhythms to environmental cues are not well-understood. Intermediate early genes (IEGs) are those genes whose transcription is rapidly induced (within minutes) by extracellular stimuli. Expression of the IEGs *c-fos* and *c-jun* have been correlated with photic entrainment of the SCN (Rea, 1989b; Aronin *et al.* 1990; Kornhauser *et al.* 1990; Earnest *et al.* 1990; Rusak *et al.* 1990; Kornhauser *et al.* 1992). Products of these IEGs, Fos and Jun, are transcription factors which dimerize and bind to AP-1 binding sites in the regulatory regions of many late response genes, leading to long-term cellular responses, which have yet to be ascertained in the SCN.

Many of these inducible genes like the IEGs, including *c-fos* (Sheng *et al.* 1990), have a sequence in their promoter region which permits transcriptional activation by second messenger-mediated signalling pathways. One example is the cAMP-response element (CRE), so named for transcriptional regulatory effects of cAMP. CRE binding protein (CREB) is a transcription activating factor which mediates the interaction between signalling pathways and CRE (Montminy *et al.* 1986; Montminy & Bilezkjian, 1987; Gonzalez & Montminy, 1989). CREB protein is phosphorylatable at several sites by a variety of kinases, including PKA, PKC, casein kinases II and IV, glycogen synthase kinase III, and calmodulin kinases (Lee & Masson, 1993). Of these sites, only phosphorylation of ser-133 appears

critical to transactivation (Gonzalez & Montminy, 1989). CREB binds to CRE sequences regardless of the phosphorylation state of ser-133, but phosphorylation must occur for enhancement of transactivation induced by extracellular stimulation (Montminy *et al.* 1986).

In mammalian cells, ligand stimulation of cell surface receptors coupled to G-proteins capable of activating adenylate cyclase results in an increase in cAMP. This increase in cAMP causes stimulation and nuclear translocation of PKA catalytic subunit, which in turn activates CREB via phosphorylation of ser-133 and promotes transcription (Montminy *et al.* 1986; Gonzalez & Montminy, 1989). Phosphorylation of ser-133 appears to promote interaction of a CREB-binding protein (CBP) with CREB, which putatively directs transcriptional machinery to the TATA box of the promoter site (Kwok *et al.* 1994; Arias *et al.* 1994). While the effects of phosphorylation of sites other than ser-133 have not been determined, it is speculated that they may enhance kinase binding to other sites in the protein, affect dimerization, or regulate DNA or co-factor binding in the CREB complex (Lee & Masson, 1993).

Recent immunocytochemical evidence indicates that light induces CREB phosphorylation at ser-133 only at times of the circadian cycle when light induces *c-fos* expression and behavioral phase shifts (Ginty *et al.* 1993). However, membrane-permeable analogs of cAMP (dibutyryl- or 8-Br-cAMP) are not capable of inducing phase shifts in the electrical activity rhythm generated by SCN in brain slices at times when the SCN is sensitive to light *in vivo* (i.e. subjective nighttime)(Gillette & Prosser, 1988; Prosser & Gillette, 1989). This suggests that another kinase may be involved in phosphorylation of CREB ser-133 upon light stimulation. Coincidentally, an analog of cGMP, 8-Br-cGMP, is

capable of causing a subjective nighttime phase-shift (Prosser *et al.* 1989). In addition, light-induced phase shifts at night are blocked by the cGMP-dependent protein kinase (PKG) inhibitor, KT-5823 (Rea, in preparation). It is well-established that PKA and PKG have functional similarities and share many substrates (Francis & Corbin, 1994), which suggests the possibility that PKG may also be capable of phosphorylating ser-133 of CREB. An early report indicated that ser-133 of Δ-CREB, a spliced variant of CREB, is not a viable substrate for PKG under physiological conditions (Colbran *et al.* 1992). However, given the coincidence of subjective nighttime sensitivities of the SCN clock to resetting by cGMP analog treatment in brain slices, light treatment *in vivo*, blockade by inhibitors of nitric oxide and PKG in photic phase shifts *in vivo*, and the light-induced phosphorylation of ser-133 in CREB in the SCN *in vivo*, we examined the possibility that CREB may serve as a substrate for PKG in the light entrainment pathway.

Methods

In vitro phosphorylation of CREBtide by PKG.

Phosphorylation of CREBtide (10 μM), a 14-amino acid sequence corresponding to amino acids 123-136 of CREB, including ser-133 (generously provided by D. Ginty and M. Greenberg, Harvard University, Boston, MA)(Ginty *et al.* 1993), was performed using either purified catalytic subunit of PKA (SIGMA) or purified PKG (Promega Co.) in the manner described in Chapter 2. Reactions were carried out in 30 μl total volume containing 20 μM Br-cGMP, using ^{32}P-ATP to monitor phosphotransferase activity. For matters of comparison, parallel reactions were carried out using similar concentrations of either

Kemptide (leu-arg-arg-ala-ser-leu-gly; SIGMA) or heptapeptide substrate (arg-lus-arg-ser-arg-ala-glu; Peninsula Labs)(Glass & Krebs, 1982), specific substrates for PKA and PKG, respectively. Reactions were carried out at 37°C for 2 minutes, then stopped with 10 μl of 1 N HCl, spotted to Whatman P81 disks, rinsed 4 times with 0.5% phosphoric acid and once with ethanol, dried, and counted.

Preincubation of phospho-CREB antiserum with PKG-phosphorylated CREBtide.

Aliquots of CREBtide were phosphorylated in tandem by equal kinase units of either catalytic subunit of PKA (2 nM) or PKG (0.5 nM) for 1 hour at 37°C. Phospho-CREB antisera provided by D. Ginty (Ginty *et al.* 1993) were then preincubated with 10X phosphorylated CREBtide (overnight, 4°C) prior to immunocytochemical localization in SCN from rats exposed to bright light for 10 minutes at a time of the circadian cycle at which light induces large phase advances in behavioral activity rhythms as well as expression of *c-fos*. Control preparations had antiserum preincubated with unphosphorylated CREBtide. Following immunostaining, an area containing the SCN was defined and immunoreactivity quantified by relative densitometry. Immunostaining and immunochemical quantitation were performed by Dr. Jian Ding.

Phosphorylation of CREB in SCN in brain slices.

In order to further evaluate the ability of SCN PKG to phosphorylate endogenous CREB, brain slices reduced in size to the borders of the SCN were stimulated by 8-Br-cGMP for 10 minutes at CT 18, a time in the circadian cycle at which 8-Br-cGMP induces large phase advances of electrical activity rhythms (Prosser *et al.* 1989). Slices were incubated in brain slice chambers for 10-12 hours, then transferred to Eppendorf tubes

containing 200 μl oxygenated EBSS at 37°C. Each sample was either 1) pretreated 10 minutes with [^{32}P]-orthophosphoric acid ($^{32}PO_4$) before 8-Br-cGMP treatment, 2) treated simultaneously with $^{32}PO_4$ and 8-Br-cGMP for 10 min, or 3) incubated with $^{32}PO_4$ for 10 minutes at 2 min, 5 min, or 10 minutes after onset of 8-Br-cGMP treatment. At the end of treatment, EBSS was aspirated and the samples frozen immediately in dry ice. Samples were then sonicated in 20-30 μl of 2X Laemmli buffer with phosphate inhibitors vanadate and fluoride, centrifuged for 5 minutes at 10,000 g, and the supernatants loaded onto 7-22% gradient SDS-polyacrylamide gels. Gels were silver stained, dried, and exposed to X-ray film for 1-10 days.

Alternatively, samples were separated on 8% mini-gels, electrotransferred onto nitrocellulose paper, and immunoblotted using phospho-CREB antiserum, using an HRP/ECL detection system.

Results

In *in vitro* reactions, heptapeptide substrate served as a slightly better substrate for PKG than did CREBtide (Fig. 12). Phosphorylation of heptapeptide substrate by PKG was inhibited significantly ($p<.01$, $n=4$) by H-8 (1 μM), an isoquinoline inhibitor specific for PKG at this concentration, and partially inhibited ($p>.05$, $n=4$) by PKI (10nM), a specific peptide inhibitor of PKA. Phosphorylation of CREBtide by PKG was inhibited significantly by both H-8 ($p<.05$, $n=4$) and PKI ($p<.05$, $n=4$). In comparison, PKA catalytic subunit was capable of phosphorylating CREBtide at a rate approximately 25% greater than an equimolar amount of Kemptide (Fig. 12), as reported previously (Colbran *et al.* 1992).

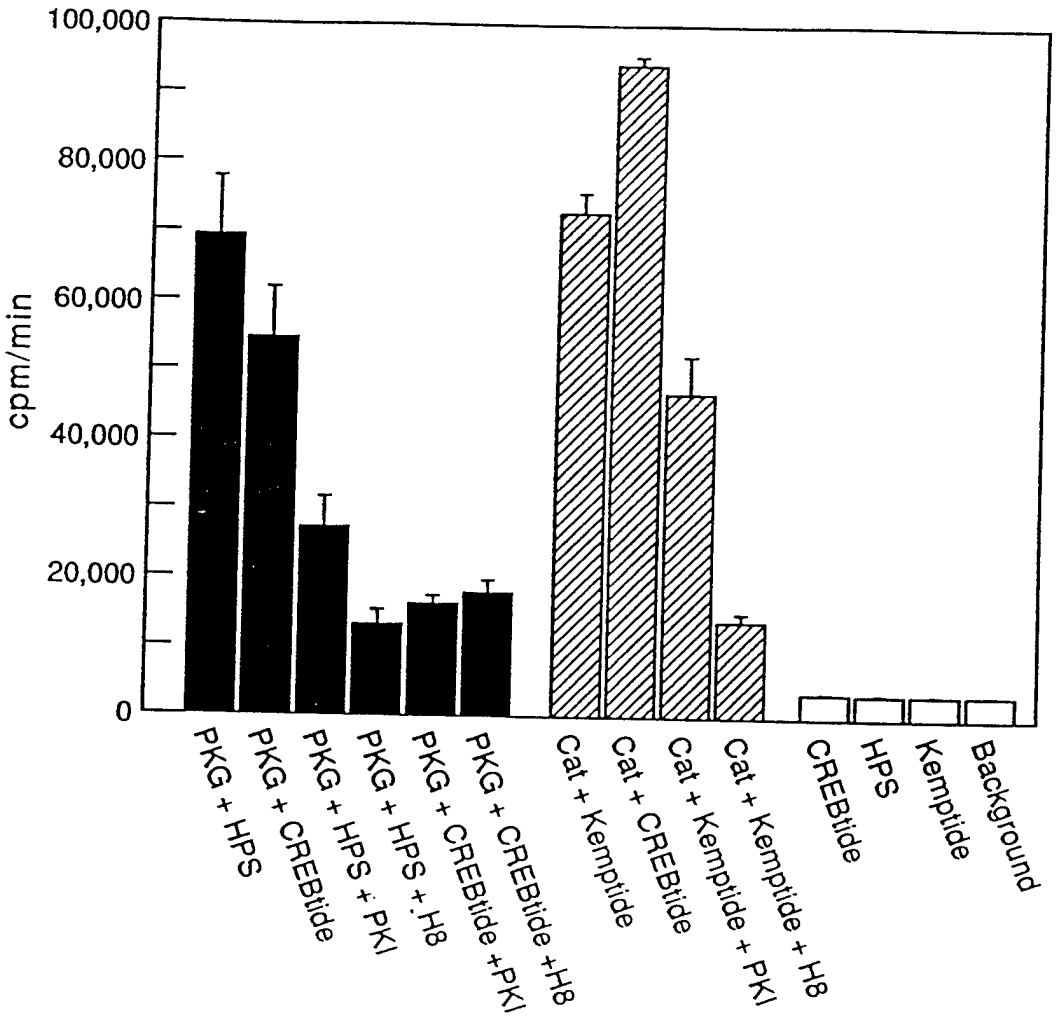


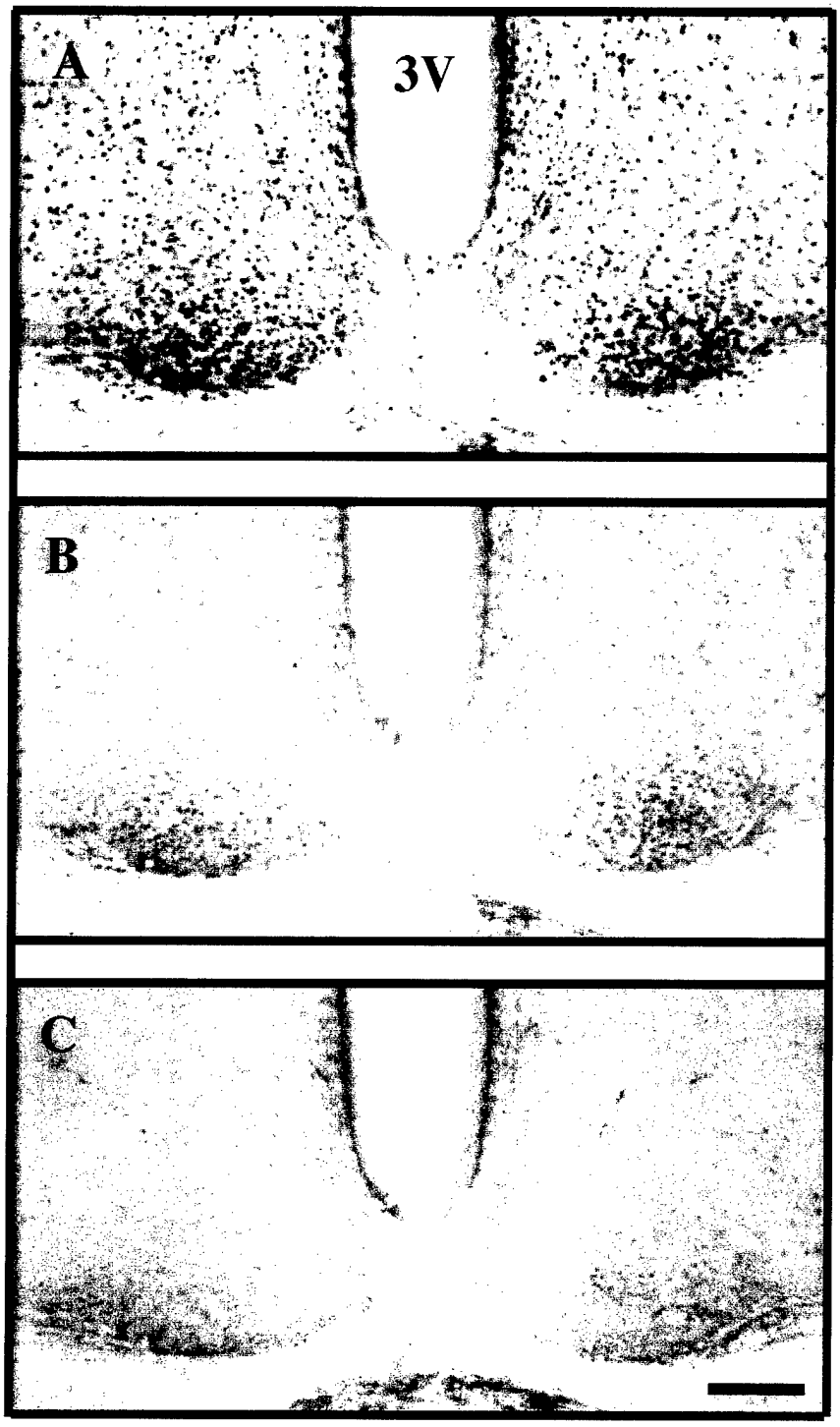
Fig. 12. Activity assays of activated PKG (solid bars, n=4) and PKA catalytic subunit (hatched bars, n=4) demonstrate affinities for CREBtide as a substrate compared to equal concentrations of their respective specific heptapeptide (HPS) and Kemptide (Kemp) substrates, respectively. Phosphorylation of CREBtide by PKG was appreciable during the 2 minute reactions, relative to HPS. Reactions catalyzed by both enzymes were decreased significantly by H-8 and PKI. Hollow bars (n=4) indicate background $^{32}PO_4$ incorporation into reaction components in the absence of kinase.

Kemptide phosphorylation by catalytic subunit was significantly blocked by H-8 ($p<.05$, $n=4$) and was reduced by PKI ($p>.05$, $n=4$). Non-specific phosphate incorporation into reaction components in the absence of kinase was not significant ($p<.001$, $n=4$) compared to any of the kinase reactions.

In the immunocytochemical study, preabsorption of phospho-CREB antiserum with CREBtide phosphorylated by activated PKG was able to significantly ($p<.01$, $n=32$) decrease light-induced phospho-CREB immunoreactivity in the SCN (86.1 ± 17.0 optical absorbance units (OAU)) by 75% (20.0 ±7.3 OAU)(Fig. 13). Pre-absorption of the phospho-CREB antiserum with CREBtide phosphorylated by PKA catalytic subunit also significantly ($p<.01$, $n=32$) reduced optical density of immunostaining in the SCN by more than 85% (11.5 ±7.1 OAU)(Fig. 13).

$^{32}PO_4$ incorporation into SCN substrates in brain slice preparations was highly variable. In 3/5 experiments, one or several protein bands in the gels demonstrated increased $^{32}PO_4$ incorporation upon 8-Br-cGMP stimulation (sample, Fig. 14), while in 2/5 experiments, no differences between stimulated and unstimulated samples were evident (data not shown). Of the bands that did demonstrate increased uptake, the 43 kD band corresponding to phospho-CREB in immunoblots demonstrated such increases (Fig. 15). However, these differences were not reflected in immunoblots of those gels with phospho-CREB antiserum (Fig. 15). In phospho-CREB immunoblots, samples displayed no increases in phospho-CREB staining (Fig. 16) after a variety of treatments (e.g., 8-Br-cGMP, S-nitroso-N-acetylpenicillamine (SNAP, a spontaneous NO generator), or glutamate) shown to produce both phase shifts of the circadian electrical activity rhythm (Ding *et al.* 1994) and

Fig. 13. Immunocytochemical staining of the SCN (A) for phospho-CREB following 10-minute treatment of light during subjective night (CT 20) was blocked by pre-incubation of the phospho-CREB antiserum with CREBtide phosphorylated *in vitro* by either (B) PKG ($p<.01$, $n=32$), or (C) PKA catalytic subunit ($p<.01$, $n=32$). Densitometry of SCN immunostaining revealed 75% and 85% decreases, respectively, in immunoreactivity from controls. Calibration bar= 200 μm. Data was collected in collaboration with J. Ding.


A
3V
B
C

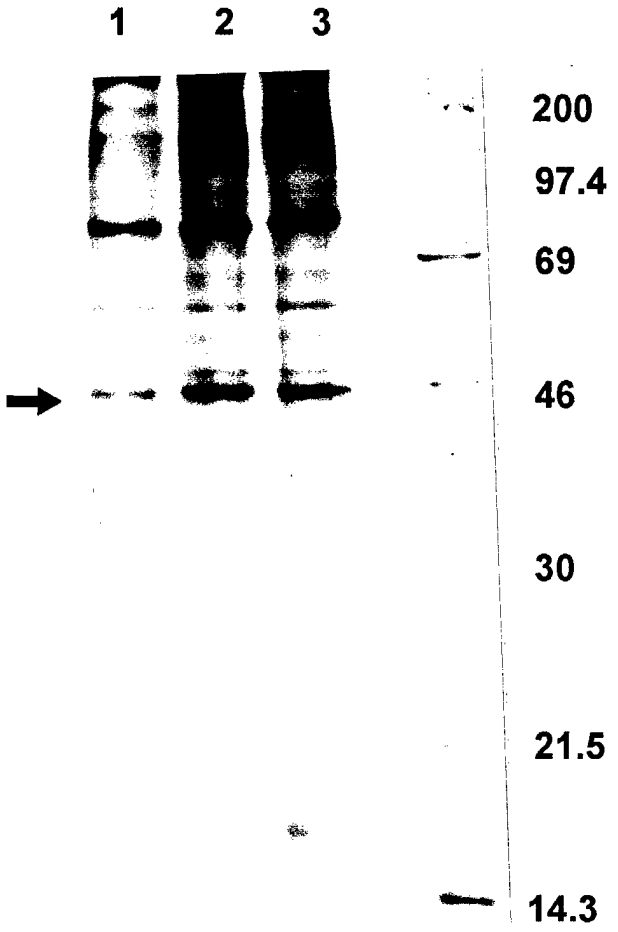


Fig. 14. In 3/5 experiments, $^{32}PO_4$ uptake increased in several bands upon stimulation of SCN in reduced slices with 2 μM 8-Br-cGMP at CT 18 (lanes 2-3). Lane 1 is a control lane which received no 8-Br-cGMP. One of the bands displaying increased uptake is a 43 kD band corresponding to phospho-CREB in immunoblots (marked with arrow). In 2/5 experiments, no differences were observed between stimulated and unstimulated SCN (data not shown).

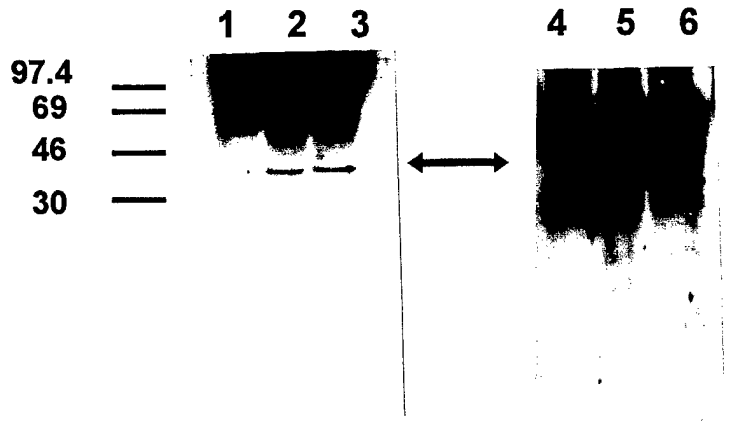


Fig. 15. (Left panel) An autoradiogram showing differences in $^{32}PO_4$ incorporation into CREB (arrow) are not reflected in (right panel) the corresponding immunoblot showing relatively equal levels of phospho-CREB (arrow) in control (lanes 1 and 4) and 8-Br-cGMP-stimulated SCN from reduced brain slices (lanes 2 and 5, 3 and 6).

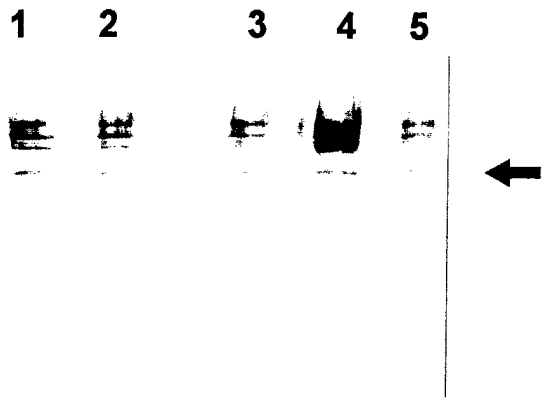


Fig. 16. An immunoblot of SCN demonstrates no noticeable differences in phospho-CREB staining from control (lane 1) despite treatments of 8-Br-cGMP (lanes 2,3), S-nitroso-N-acetylpenicillamine (SNAP, a nitric oxide donor)(lane 4), or glutamate (lane 5), all of which have been shown to produce phase shifts and increased phospho-CREB (ser-133) immunocytochemical staining (Ding, unpublished data).

concommitant CREB phosphorylation in the SCN at this circadian timepoint, demonstrated by immunocytochemical staining (Ding, unpublished data). Neither varying onset of $^{32}PO_4$ incubation relative to onset of 8-Br-cGMP stimulation, nor increasing duration of $^{32}PO_4$ incubation incrementally from 2-10 minutes (Fig. 17) resulted in noticeable differences in uptake.

Discussion

The hypothesis that the light entrainment pathway acts through PKG to phosphorylate CREB gains support from the present series of experiments. While CREBtide serves as a better substrate for PKA than for PKG in *in vitro* reactions, PKG phosphorylates ser-133 at a considerable rate. Several considerations must be made when evaluating the physiological significance of this observation. First, the rate of the PKG-mediated phosphorylation of CREBtide is not significantly lower than the rate of the phosphorylation of heptapeptide, which has been used to characterize the kinetics of PKG. Differences in values obtained here and those reported previously may be due to differences in reaction components or temperature differences between protocols. Although the rate of PKG-mediated phosphorylation of CREBtide is lower than that by PKA, it is comparable to the rate of phosphorylation of the PKG-specific peptide substrate used in prior characterizations, which have been interpreted to reflect kinetics of PKG activity *in vivo*.

Secondly, the kinetics of this PKG-CREBtide reaction may not accurately reflect endogenous reaction of PKG with the complete α-CREB protein. As discussed by Colbran *et al.* (Colbran *et al.* 1992), catalytic efficacy of PKG is diminished by cleavage of amino acid

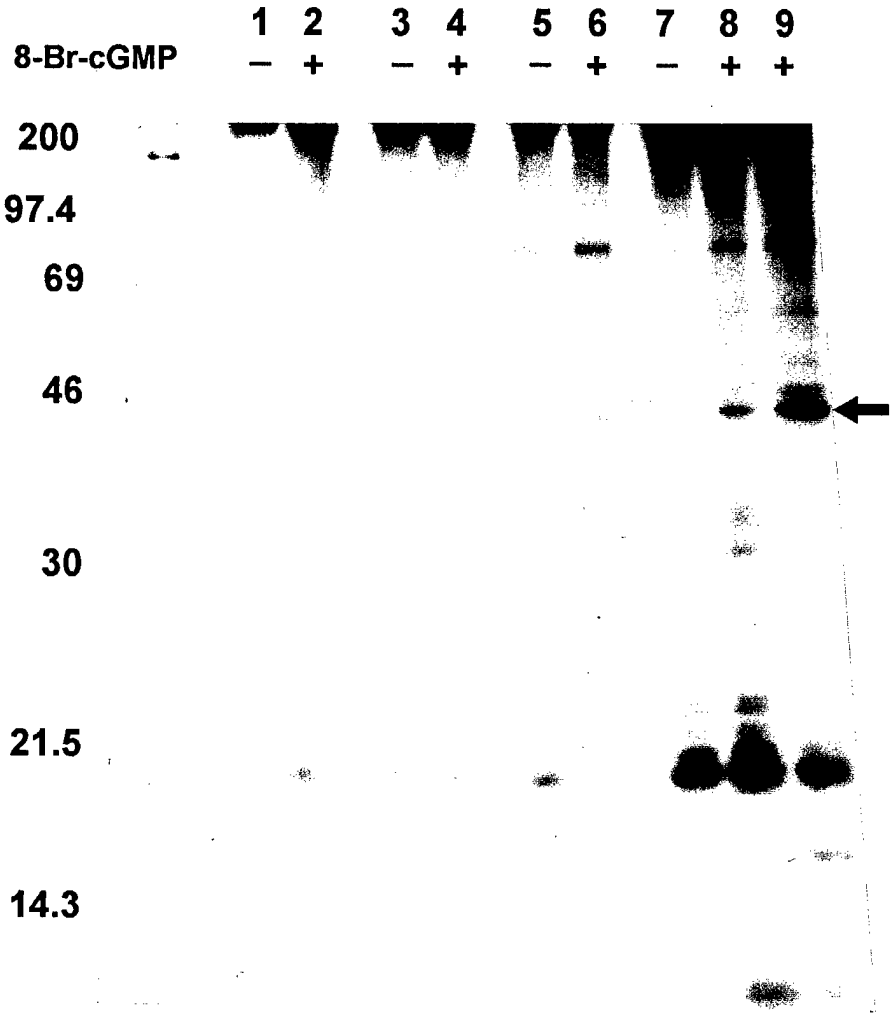


Fig. 17. Length of incubation of reduced brain slices with $^{32}PO_4$ affected only intensity of incorporation into CREB in control (-) and cGMP-stimulated (+) SCN. Samples were incubated for 1 (lanes 1,2), 2 (lanes 3, 4), 5 (lanes 5, 6), or 10 (lanes 7-9) minutes. Notice the intense incorporation into CREB (marked with arrow) 10 minutes after treatment with 8-Br-cGMP.

sequences carboxy-terminal to phosphorylated residues. Thus, the brevity of the carboxy terminus following the phosphorylated serine of CREBtide may contribute to the relatively lower rate of phosphorylation of CREBtide by PKG versus that by PKA catalytic subunit. Other factors, such as specific effects of amino acids in substrate sequence, or differences in secondary or tertiary structures, may also influence kinase specificity and affinity (Colbran *et al.* 1992). Thus, inferences drawn from PKG interactions with CREBtide or Δ-CREB are, at best, tenuously applied to PKG interaction with endogenous α-CREB. This author contends that the observations herein merely indicate the possibility of a PKG-mediated phosphorylation of ser-133 of CREB based on the observation that CREBtide will bind to the catalytic site and serve as a substrate of PKG. While PKG phosphorylates Δ-CREB, a spliced variant of α-CREB, at a rate much slower than PKA (Colbran *et al.* 1992), there remains the possibility that the kinetics of this reaction *in vivo* may be affected by interactions of CREB and/or PKG with other co-factors which bind to that complex. Alternatively, the prolonged activation of PKG by autophosphorylation or extended presence of cGMP within the cell may allow a degree of phosphorylation of CREB sufficient to alter transcription, despite the relatively slower rate of reaction.

The hypothesis that CREB serves as a substrate for PKG in the light entrainment pathway is strengthened by the present demonstration that PKG-phosphorylated CREBtide can block specific phospho-CREB immunostaining in SCN at a time in the circadian cycle (CT 20) when photic stimulation typically results in CREB phosphorylation. The degree to which PKG-phosphorylated CREBtide was able to block immunostaining was comparable to that of PKA catalytic subunit-phosphorylated CREBtide prepared under similar

conditions. Thus, it can be asserted that the CREBtide sequence is phosphorylatable by PKG as well as PKA, rendering it recognizable by specific phospho-CREB antibodies. However, this added evidence still may not reflect actual intracellular kinetics and thus the probability of PKG-mediated phosphorylation of CREB in the intracellular environment.

In order to examine the process of PKG-mediated phosphorylation of CREB in a more natural environment, $^{32}PO_4$ incorporation into substrates resulting from cGMP analog stimulation of semi-intact SCN in reduced brain slices was examined. Incorporation of $^{32}PO_4$ into proteins of the SCN was evident after 2 minutes exposure. Longer incubation tended only to increase amount of phosphate incorporated. The fact that phospho-CREB immunoblot staining did not differ among treatments suggests that either differences are not quantifiably measureable, or alternatively, the differences in $^{32}PO_4$ incorporation seen in the autoradiograms reflects phosphorylation of sites on CREB other than ser-133, which would not be evident in the immunoblots. Immunocytochemical staining of unstimulated SCN do demonstrate a background level of phospho-CREB staining, so it is not surprising that some $^{32}PO_4$ incorporation is evident in unstimulated samples in the immunoblots. However, in control immunoblot experiments with other substrate-antibody reactions, two-fold increases in substrate concentration were measureable (Weber & Gillette, 1994). This suggests that either several-fold differences in ser-133 phosphorylation are not measureable due to characteristics of this particular antiserum, or that CREB phosphorylation is occuring as an artifact of experimental procedure. Attempts to reduce background staining for phospho-CREB by controlling for phenomena which might stress the tissue during phosphorylation

reactions and thus induce CREB phosphorylation (tissue handling, temperature fluctuations, pH changes) have been unsuccessful.

The possibility that increased $^{32}PO_4$ incorporation may reflect phosphorylation of sites other than ser-133 was considered. The probability that kinases other than PKG (with the exception of PKA) are stimulated by 8-Br-cGMP is unlikely. However, in order to control for this possibility, as well as to attempt a reduction in non-PKG-specific (background) incorporation, a mixture of inhibitors for several prominent kinases in brain tissue which have been shown to phosphorylate sites in addition to ser-133 on CREB were applied prior to phosphate incorporations. Preincubation of slices with a mixture of KT-5720 (0.02μM, @-PKA), KN-62 (0.5 μM, @-Ca^{++}/calmodulin (CaM) kinase), genistein (10 nM, @-tyrosine kinase), and calphostin (0.5 μM, @-PKC), was expected to lower background phosphorylation within samples, in particular the non-ser-133 phosphorylation of CREB, which may be masking the effects of PKG. No appreciable differences were seen with addition of these inhibitors to unstimulated samples (n=2; data not shown), suggesting that background incorporation into proteins in the samples is either a result of activity of other kinase(s) not inhibited by these compounds, or is non-specific exchange of phosphate into SCN proteins. These possibilities could be addressed with experiments employing other kinase inhibitors, or possibly by manipulating length of incubation with $^{32}PO_4$ and specific activity of the radiolabelled phosphate.

In summary, evidence presented here suggests that PKG may phosphorylate and transactivate CREB, as 1) CREBtide is phosphorylated by PKG, though slower than by PKA, at a rate comparable to that for the PKG-specific heptapeptide substrate, and 2)

CREBtide phosphorylated by either PKG or PKA catalytic subunit is capable of blocking immunocytochemical localization of light-induced phospho-CREB in the SCN. In addition, $^{32}PO_4$ incorporation into a 43 kD band from the SCN which corresponds to phospho-CREB in immunoblots often increases following 8-Br-cGMP stimulation of the SCN in reduced brain slices; the reason for the inconsistency of this observation is still not evident. Given these data, the physiological relevance of relatively slower, but feasible, phosphorylation of CREB by PKG should be further evaluated.

CHAPTER 5. SUMMARY.

The SCN are the primary circadian pacemaker. The SCN can be studied in the brain slice, in isolation from neural projections which function to modulate the activity of these nuclei. The intracellular messenger, cGMP, was demonstrated to oscillate over the course of the circadian cycle, in synchrony with the activity and autophosphorylation state of PKG, implying a role in SCN function. While the mechanisms responsible for this oscillation in cGMP levels is not yet known, the regulation of PKG activity may impart the temporal sensitivity of the SCN to the resetting effects on circadian rhythms seen in previous studies. Quite possibly, the biochemical machinery responsive to cGMP is "ready and waiting" for the endogenous rise in cGMP levels, which then begin a cascade of kinase-regulated effects which complete the cycle. The exact role of PKG has yet to be determined.

Nitric oxide was demonstrated to play a role in resetting the circadian pacemaker in response to photic stimuli. Phase shifts in locomotor activity in response to light pulses during the subjective nighttime in hamsters were blocked by inhibiting the enzyme responsible for NO production, implicating NO in the signal transduction pathway from the retina to the SCN. Inhibition of nitric oxide synthase did not block photic induction of the *c-fos* gene product, Fos, which has been correlated previously with photic phase-shifts. This result, the first to demonstrate blockade of phase shifts without blockade of Fos production, suggests that either Fos is produced upstream of nitric oxide synthesis, or that Fos is produced in response to photic stimuli by a biochemical pathway parallel to the nitric oxide-dependent pathway. It is not unlikely that light signals are transduced via multiple signal transduction pathways, and that activation of two or more of these pathways are necessary for full expression of phase shifts.

Recently, phosphorylation state of the transcription factor, CREB, has been correlated with photic phase shifts. In the experiments described here, it was demonstrated that CREB is a putative substrate for PKG. Several lines of evidence support this hypothesis. First, CREBtide, a 14 amino acid peptide sequence of CREB including the phosphoacceptor serine (133) responsible for enhanced activation of the cAMP response element, is phosphorylatable by PKG *in vitro*. Secondly, phosphorylation state of CREB in the SCN in brain slices is seen to increase following stimulation by 8-Br-cGMP. Thirdly, PKG-phosphorylated CREBtide is capable of blocking phospho-CREB immunostaining of SCN from animals subjected to phase-shifting pulses of light during subjective nighttime.

Together, these data support the following model of a hypothetical light input pathway to the SCN, modified from the original model proposed in the introduction. In it, retinal illumination results in glutamate release from direct retinal projections stimulates NMDA receptors in the SCN. This activates nitric oxide synthase via an increase in intracellular calcium. The resulting NO stimulates an increase in cGMP through activation of soluble guanylate cyclase and subsequent activation of PKG, which is required at least for phase advances. PKG may then go on to phosphorylate CREB, enhancing its ability to induce transcription. Since cGMP results only in phase advances ($\phi+$) of the electrical rhythms in SCN in brain slices at times when light (*in vivo*), glutamate and nitric oxide (*in vitro*) produce phase delays ($\phi-$), it is likely that another pathway activated by NO, acting either independently of or through interactions with phase advance mechanisms, results in phase delays.

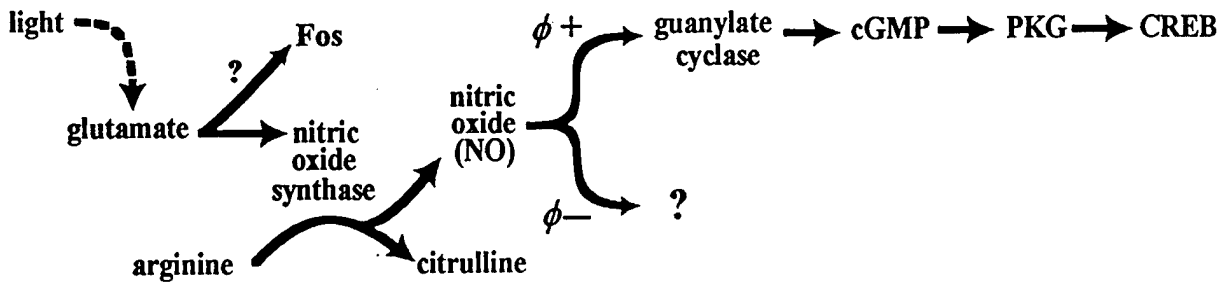


Fig. 18. Proposed model for signal transduction of photic information into the SCN.

Many studies remain in elucidation of photic phase-shifting. While key players have been identified, primarily by inhibiting activity or production of specific molecules, agonist effects have been ineffective at mimicking the effects of light when injected *in vivo*, warranting evaluation of regulation of the light input pathway by other projections to the SCN.

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VITA

E. Todd Weber entered this world on March 7, 1966, in New Castle, Pennsylvania. He attended Mohawk Elementary and Mohawk High Schools, and upon graduation, embarked on a journey into the world of academia (the first in his family, don't you know) at Slippery Rock University, in Pennsylvania. Working summers in the Ohio Valley's mills and machine shops, he soon realized that any fortune he might achieve would be in the realm of scholarliness, so on he pushed, to obtain his Bachelor's of Science degree in Biology, in 1988. He then continued his academic career by following a long line of Slippery Rock University students destined to become physiologists at the University of Illinois at Urbana-Champaign. Having arrived at the U of I fresh from a summer internship at Smith, Kline and French Pharmaceuticals near London, he was welcomed into the lab of the awe-inspiring C. Ladd Prosser to continue his quest to reveal the source of intrinsic rhythmicity in the gastrointestinal tract. After a year of electrophysiology, he was once again enlightened by Fate, realizing that he was destined to appreciate electrophysiology but clutch the concreteness of biochemistry to his bosom. He was then allured into another oscillatory field of physiology, that of circadian rhythms. Drafted into the graces of the famous woman scientist, Dr. Martha Gillette, he hitched his drawers and jumped headlong into the obscure understanding of cGMP and its dependent kinase, and the role they play in circadian rhythm generation by the suprachiasmatic nucleus in the rat. Armed only with an Air Force Graduate Research Fellowship and a keen sense of humor, he ferreted out the involvement of nitric oxide in SCN function, with the help of Air Force *scientist extraordinaire*, Dr. Michael Rea, with whom he intends to soon embark on another leg of his journey.