Modulation of synaptic transmission in the suprachiasmatic nucleus

by

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CERTIFICATE OF APPROVAL

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LIST OF FIGURES

ABBREVIATIONS

4-AP: 4-aminopyridine
5-HT: 5-hydroxytryptamine
ACSF: artificial cerebrospinal fluid
AMPA: (RS)-α-Amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid
APV: DL-2-Amino-5-phosphonopentanoic acid
AVP: arginine-vasopressin
cAMP: cyclic adenosine monophosphate
CCD: charge coupled device
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione
CREB: cAMP-response element binding protein
CT: circadian time
DSI: depolarization-induced suppression of the inhibitory current
EGTA: ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EPSCs: excitatory postsynaptic currents
F: fluorescence intensity units
GABA: γ-aminobutyric acid
GAD: glutamic acid decarbosylase
GRP: gastrin-releasing peptide
Ih: hyperpolarization-activated current
IPSC: inhibitory postsynaptic current
IR-DIC: infrared differential interference microscopy
mEPSC or mIPSC: miniature excitatory (inhibitory) postsynaptic currents
NEM: N-ethylmaleimide
NMDA: N-methyl D-aspartic acid
N/OFQ: nociceptin / orphanin FQ
NPY: neuropeptide Y
ORL1: opioid receptor like receptor 1
PACAP: pituitary adenylate cyclase activating protein
PPD: paired-pulse depression
PVN: paraventricular nucleus
RHT: retinohypothalamic tract
SCN: suprachiasmatic nucleus
SSTR: somatostatin receptor
TTX: tetrodotoxin
UFP-101: [Nphe(1), Arg(14), Lys(15)]N/OFQ-Nh(2)
VIP: vasoactive intestinal polypeptide
ZT: zeitgeber time

Abstract

Endogenous circadian rhythms in mammals are controlled by neurons located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Many SCN neurons express a day / night rhythm in physiological processes that influence inter-neuronal synaptic communication, including the action potential firing frequency and the expression patterns of proteins involved in neuronal communication. In this work, I explore several modulatory mechanisms in the synaptic circuitry of the SCN that ultimately help form or sustain its circadian rhythm and output. The overriding concern of this thesis is to explore the mechanisms by which fast synaptic transmission is modulated to convey relevant temporal information over the 24-hour cycle. In order to understand these mechanisms, I have used acute brain slices and electrophysiological recording techniques to examine synaptic plasticity, retrograde inhibition and neuropeptide inhibition of synaptic transmission in the SCN.

I have found (Manuscript #1) a significant diurnal rhythm in the synaptic strength of intra-SCN GABAergic synapses by examining evoked pairs of currents. Short-term synaptic depression is an activity-dependent reduction in the strength of synaptic transmission that occurs within several hundred milliseconds of the onset of activity and acts as a temporal firing frequency "filter" in intercellular neurotransmission. In the SCN, two forms of short-term synaptic plasticity correlate well with the diurnal cycle. The data suggest that this mechanism is an integral part of intra-SCN synaptic neurotransmission conveying temporal information between cells.

I have also found (Manuscript #2) that a subset of SCN cells uses a retrograde signal to inhibit synaptic input. This involves postsynaptic depolarization-induced Ca^{2+}

entry, which stimulates the release of a factor from the postsynaptic cell that inhibits presynaptic GABA release. The inhibition is synapse-specific and can occur at action potential firing frequencies observed in the SCN. Although retrograde inhibition was observed in cells recorded during both the day and the night, cells in the SCN are more likely to fire action potentials at these frequencies during the subjective day. Thus, a daytime modulation of synaptic communication is possible in the SCN.

Third (Manuscript #3), a detailed examination of the action of the peptide Nociceptin / Orphanin FQ (N/OFQ) on synaptic activity in the SCN was performed. Previous data (Allen et al., 1999) show N/OFQ binding is very high in the SCN and a large majority of cells respond directly to the peptide. I show that these inhibitory effects, along with an inhibition of the vesicular release machinery, inhibit presynaptic GABA release. Previous work also shows that light-induced advances in hamster circadian rhythms are inhibited by N/OFQ (Allen, et al., 1999). Here, I present data that N/OFQ presynaptically inhibits the glutamatergic terminals responsible for light inputs by inhibiting both Ca^{2+} dynamics and the vesicular release machinery. Thus, N/OFQ can be considered an important inhibitory regulator of synaptic communication in the SCN, leading to reduced sensitivity to environmental light inputs.

Overall, these three mechanisms provide the circadian system with significant control over fast synaptic neurotransmission. This modulatory control can be viewed as instrumental in the information processing between SCN neurons and from environmental inputs. A rich variety of mechanisms of transmitting circadian information is available to the SCN, including diffusible factors and gap junctions discussed below. Here, I examine the concept that through modulatory mechanisms, fast synaptic neurotransmission can convey information relevant to the 24-hour long course of the circadian cycle.

Introduction

Circadian Rhythms

The 24-hour environmental light/dark cycle provides one of the most important environmental signals to an organism's vital functions. To correctly respond to and anticipate the daily cycle, almost all organisms exhibit approximately 24-hour behavioral and physiological rhythms. These rhythms are termed "circadian"—from the Latin "circa diem" meaning "about a day"—and are endogenous, self-sustaining rhythms of biological activity that persist in the absence of external time cues. An internal clock generates and coordinates this timekeeping system. In the absence of environmental time cues, the internal clock free runs with a phase that is usually close to but not precisely 24 hours. Environmental cues, most notably light, act as controlling stimuli to synchronize—or "entrain" (originally from the French entrainer, "to carry along")—the endogenous clock to the 24-hour light/dark cycle. These entraining stimuli are termed "Zeitgeber"—translated from German as "time-giver"—stimuli and serve to reset the endogenous circadian rhythm. Ultimately, Zeitgeber cues allow a stable phase relationship between the endogenous "circadian time" and the environmental light/dark cycle. The success of synchronization affects survival in some species, such as nocturnal rodents that can become prey during daylight, and health and well-being in others, such as the impaired cognitive function, altered hormonal function, and gastrointestinal complaints experienced by humans.

It is thought that a selective evolutionary advantage that has been passed down to humans is conferred by the ability to express circadian rhythms and synchronize these with environmental stimuli (DeCoursey et al., 1997; Ouyang et al., 1998). Most

civilizations have documented the passage of time in one way or another, indicating its importance in human life. In humans, circadian rhythms are found in the secretion of hormones such as melatonin, growth hormone, thyroid-stimulating hormone and cortisol, and the urinary secretion of potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and water, while physiological parameters such as blood pressure and core body temperature also vary (for review, see (Cermakian and Boivin, 2003)).

Human circadian rhythms are further influenced by numerous factors beyond light inputs, such as social interactions (Honma et al., 2003). Societal demands are often in conflict with the human circadian clock and can influence human health through abnormal sleep patterns and certain forms of human depression (Bunney and Bunney, 2000). Moreover, disasters such as the Chernobyl or Three Mile Island nuclear power plant emergencies and the Exxon Valdez spill all occurred at the low point of the worker's circadian efficiency and temperature cycle. Current U.S. employment figures demonstrate that up to 20% of the population works during non-day shift hours. Problems can arise either while the employee is shifting from daytime activity to nighttime activity—as is the case with rotating shifts—or when the night shift employee spends time with friends and family who do not work nights (Monk, 2000). Together, these problems demand a better understanding of the internal clock's mechanisms.

The mammalian Suprachiasmatic Nucleus (SCN)

In mammals, the master internal timekeeping system is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, two tightly packed groups of 8,000 – 10,000 cells each, located on either side of the third ventricle immediately dorsal to the optic chiasm. The SCN was clearly identified as the locus of the master clock by early

ablation and transplantation studies (Moore and Eichler, 1972). Ablation of the SCN leads to the loss of endogenous rhythmicity as recorded by the free-running circadian rhythm of wheel running activity in hamsters held in constant darkness (Stephan and Zucker, 1972). Transplantation of cells from the SCN of an intact animal into the lesioned animal imposes the circadian rhythmicity of the donor animal onto the lesioned animal (Lehman et al., 1987; Ralph et al., 1990; Silver et al., 1990). One question that rises from these studies is how this group of cells produces a circadian rhythm in mammals.

The SCN acts as an ensemble of individual oscillators

Even unicellular organisms express rhythms similar to mammals, demonstrating that circadian rhythms are generated at the cellular level (Kondo et al., 1993; Lin et al., 1999; Mittag and Wagner, 2003). In individual SCN cells, the expression of so-called clock proteins cycle in double autoregulatory feedback loops to regulate their own transcription in a circadian manner (recent reviews include: Reppert and Weaver, 2002; Panda et al., 2002b; Okamura, 2003). A circadian rhythm of action potential firing frequency can also be measured in many individual SCN cells when dispersed in cell culture (Welsh et al., 1995).

How then do these individual oscillators communicate with each other to construct the resulting circadian rhythm that controls organismal rhythms? In the mammalian SCN, the sum average of these individual oscillations can be measured in numerous ways. For instance, one can follow the peak and nadir of ensemble electrical excitability as expressed by the action potential firing frequency both *in vivo* and in the brain slice preparation *in vitro* (Inouye and Kawamura, 1979;Green and Gillette,

1982;Walsh et al., 1992). These studies demonstrate that the circadian rhythmicity of the individual oscillators as well as of the ensemble SCN is maintained in a brain slice preparation. A few models that help address the intercellular coupling that brings about this coordinated circadian rhythm within the SCN have been proposed. One study, using animals and SCN tissue cultures from animals expressing mutation-induced variations in circadian period length (Liu et al., 1997) found that though individual clock cells from dispersed SCN culture vary greatly in their period length, the average periods correlate well with the whole-animal period. Given that dispersed cells vary more than cells in organotypic culture, it was deduced that intra-SCN cell coupling is responsible for keeping SCN cells synchronized, even though each autonomous cellular period is determined by its own molecular clock. A model was proposed in which the circadian phases of core oscillators in the SCN are weakly coupled to one another and this core then recruits outlying cells to oscillate under the same period. Eventually, the majority of cells in the SCN oscillate with a period close to the average period expressed by the whole organism (Reppert and Weaver, 2002). More recently, a study examining acute primary brain slice cultures described the SCN as a network of at least three separate groups of oscillators, the phases of which distribute around the average phase of the entire network (Quintero et al., 2003). This phase heterogeneity, it was hypothesized, could arise from intercellular coupling. The relationship between these oscillators can be modulated by environmental input, a conclusion that is consistent with other models in which rhythmic and non-rhythmic regions of the SCN are delineated by the presence or absence of cells expressing calbindin and cells receiving retinal innervation (Antle et al., 2003). This last model depends upon rhythmic output neurons and nonrhythmic "gate" populations. The "gate" neurons help reset the circadian phase of the entire

network, either following environmental input or by the output signals themselves, once a threshold of output activity has been achieved.

All three of these models incorporate the concept of intra-SCN neuronal coupling. In the first model, cellular coupling is of paramount importance to maintain the synchronized rhythmic oscillations of the SCN (Reppert and Weaver, 2002). The second model allows for cellular coupling and a range of sensitivity to the coupling signal to achieve the same end (Quintero, Kuhlman, and McMahon, 2003). And though the third model does not depend on coupling, it allows that cellular coupling could decrease the variability of period length among oscillators (Antle, et al., 2003).

What sorts of intercellular coupling mechanisms are available to SCN cells? Coupling leading to the expression of some circadian rhythms, such as hamster wheel-running activity, may be achieved by synapse-independent mechanisms. More specifically, SCN cells transplanted into SCN-lesioned arrhythmic animals restore the circadian rhythmicity of wheel-running activity even when the SCN cells were transplanted into the third ventricle (LeSauter et al., 1997) and when the SCN cells were dispersed and subsequently encased in an sylastic tube to inhibit synapse formation (Silver et al., 1996). Another indication of synapse-independent coupling is that blockade of intra-SCN synaptic neurotransmission for a period of days does not interfere with phase coherence, as shown by the faithful re-establishment of the appropriate phase following blockade relief. Several alternative coupling pathways have therefore been proposed, such as gap-junctions (Ueda and Ibata, 1989; Jiang et al., 1997b; Colwell, 2000; Jobst et al., 2004) and diffusible factors. Transforming growth factor-alpha (Kramer et al., 2001) and prokineticin 2 (Cheng et al., 2002) are two putative diffusible factors.

The discovery that diffusible factors influence circadian locomotor behavior, however, does not indicate that diffusible factors are the primary coupling mechanism of circadian rhythms, either between SCN neurons or for the coordination of rhythms to the various output target areas. To illustrate how diffusible factors and synaptic communication both play a role in coordinating and shaping circadian rhythms, consider what is known about a particular output target area of the SCN. Numerous projections from the SCN to target areas involved in neuroendocrine and autonomic control are believed to underlie organismal control of circadian rhythmicity (Reppert and Weaver, 2002). The classic example of this is the multi-synaptic pathway regulating melatonin release. GABA- and glutamatergic efferents from the SCN project to cells in the paraventricular nucleus (PVN), which, in turn, projects to the intermediolateral nucleus of the upper thoracic spinal cord. These, in turn, project via the superior cervical ganglia to the pineal gland, which is the site of melatonin production and release (Larsen et al., 1998). Another functional influence by the projection to the PVN is the circadian rhythmicity of adrenal steroids corticosterone and cortisol (Kalsbeek et al., 1996). An ablation-transplantation study showed that, in hamsters with restored circadian locomotor activity following transplantation, neither melatonin nor the adrenal steroids cycled in a circadian manner, suggesting that synaptic output from the SCN is required for these processes, even if locomotor activity can be restored without synaptic output (Meyer-Bernstein and Morin, 1996). These findings were strengthened by data showing that blockade of SCN – PVN synaptic transmission can induce daytime melatonin secretion (Kalsbeek et al., 2000). However, a recent study shows that arginine-vasopressin (AVP) can act as a diffusible factor from the SCN to generate a circadian rhythm in action potential firing frequency in the PVN recorded from

hypothalamic slice cultures in which the SCN is excised (Tousson and Meissl, 2004). Yet the study also shows that AVP does not have the same effect in intact brain slices, indicating that under these conditions the neuronal (synaptic) signal overrides the humoral signal from the SCN.

Similarly in the SCN itself, diffusible humoral signals, or regularly released peptides, may serve to consolidate the broad circadian rhythms of SCN cells even in the absence of synaptic neurotransmission—as described above where synaptic transmission was blocked while the circadian clock continued to function. But, the network itself is a circuit of inhibitory synaptic connections that presumably functions as a more immediate cell-to-cell communication mechanism. This may influence other processes—for example action potential firing patterns—critical for the coordination between SCN neurons and the projection of a cohesive output pattern to target areas.

Intercellular communication in the SCN

In the SCN, nearly all neurons contain the γ-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD), and many synapses have been shown to contain GABA (Moore and Speh, 1993). Inhibitory GABAergic synapses connect the local neuronal circuitry via post-synaptic $GABA_A$ receptors (Kim and Dudek, 1992; Strecker et al., 1997; Jiang et al., 1997a). The notion that GABA is a coupling signal in the SCN comes from observations that individual, dispersed SCN neurons that drift out of phase can be synchronized by GABA (Strecker, et al., 1997; Liu and Reppert, 2000). Also, when these cells do form synaptic connections, individual cells can synchronize action potential firing using these connections (Honma et al., 2000; Shirakawa et al., 2000). These results demonstrate that intercellular synaptic

communication via $GABA_A$ receptors is an important mechanism coupling SCN neurons.

Intercellular coupling mechanisms enable the SCN to generate behavioral or physiological phase shifts in response to entraining signals. An indication that synaptic GABAergic signaling plays a role in this process comes from data showing that bicuculline, a $GABA_A$ antagonist, blocks the phase delay of light pulses administered during the early night, while it has no effect on phase-advancing light pulses during the late night (Ralph and Menaker, 1985). And, during the subjective day, muscimol, a $GABA_A$ agonist, produces phase advances when microinjected into the SCN of nocturnal hamsters (Smith et al., 1989), but phase delays when injected into the diurnal grass rat (Novak and Albers, 2003). It has also been shown that glutamate-mediated phase shifts can be blocked by prior administration of muscimol (Gillespie et al., 1996; Mintz et al., 2002), and $GABA_A$ receptor activation may be required in the control of arousal-induced, serotonin-mediated phase-shifting stimuli (Mintz et al., 1997). These results indicate an important role for intra-SCN GABAergic synapses in coupling SCN neurons following phase-shifting stimuli. The fact that the responses to these phase-shifting stimuli vary with circadian time suggests the possibility that interneuronal synaptic modulation may play a role in these processes.

A prominent feature of SCN physiology that influences and can also be influenced by interneuronal synaptic neurotransmission is the action potential firing frequency. Neuronal excitability, as measured by the firing frequency, peaks during the subjective day and exhibits a nadir during the subjective night in both nocturnal and diurnal species (Inouye and Kawamura, 1979; Green and Gillette, 1982; Walsh, et al., 1992). Assuming that the action potentials recorded from the soma are faithfully

transmitted to the axon terminals, it is likely that daytime intra-SCN GABAergic communication peaks at frequencies between 2 and 22 Hz, with an average frequency of 5 - 7 Hz (Groos and Hendriks, 1982; Welsh, et al., 1995;Jiang, et al., 1997a; Pennartz et al., 1998). The action potential firing rate, which presumably controls the rate of fast synaptic neurotransmission, could therefore in and of itself reflect the relative circadian phase of the presynaptic cell by the frequency of GABA release. In other words, more frequent synaptic inputs from neighboring cells could serve as the light-phase signal versus less frequent synaptic transmission during the dark phase. However, recent studies have found that spontaneous IPSC activity either does not vary during the circadian cycle (Lundkvist et al., 2002) or peaks during the subjective night in the dorsomedial portion of the SCN (Itri and Colwell, 2003). If this is an accurate representation of intra-SCN communication *in vivo*, it may indicate that significant modulation of synaptic activity occurs in the SCN to attenuate the transmission of action potential firing frequency at the axon terminal. In support of this possibility, recent evidence suggests a circadian rhythm in synaptic vesicle docking machinery (Panda et al., 2002a) that might be responsible for altering intra-SCN synaptic neurotransmission. At the postsynaptic side of the synapse, dendritic vesicles have been found (Castel et al., 1996), which allow for the possibility of retrograde modulation of synaptic neurotransmission in the SCN.

The above data suggest that intercellular communication between SCN neurons is important for the generation and environmental synchronization of circadian rhythms and that modulatory mechanisms may play a role to help shape these rhythms. Work presented in this thesis is concerned with examining the modulatory mechanisms of synaptic communication in the SCN. The mechanisms examined using

electrophysiological techniques may each affect synaptic neurotransmitter release in response to somatic action potentials. One hypothesis examined is whether synaptic strength itself is constant over a range of possible transmission frequencies and over the course of the circadian cycle. Another mechanism of synaptic modulation examined involves the postsynaptic cell directly inhibiting transmitter release from presynaptic cell terminals through a process termed "retrograde inhibition". A third mechanism of synaptic modulation is through neuromodulatory peptides activating presynaptic receptors. More specifically, I have examined the hypothesis that N/OFQ acts to inhibit GABAergic and glutamatergic neurotransmission. These three possible types of synaptic modulation are discussed in more detail below.

Synaptic Modulation

Individual synaptic events—meaning the release of neurotransmitter and the subsequent postsynaptic current and voltage responses—are relatively short-lived, on the order of milliseconds, compared to the 24-hour clock. I have therefore concentrated on three types of modulation of synaptic transmission in the SCN that each could affect the strength of the transmitted signal over longer periods of time, and therefore could confer a greater relevance of fast synaptic transmission to the circadian clock. The three types of synaptic modulation can roughly be divided by their sites of action and by the time frames within which they are effective. The first form of modulation, short-term synaptic plasticity, is an exclusively presynaptic event and acts on the order of hundreds of milliseconds as recorded from pairs of evoked inhibitory postsynaptic currents (IPSCs). Second is retrograde inhibition, in which postsynaptic depolarization triggers a Ca^{2+}-dependent release of retrograde transmitter that acts presynaptically for a number

of seconds. Third, the peptide N/OFQ is likely released from a projection—candidates to be discussed in the discussion—into the SCN and has a timecourse of action that is limited only by its presence in the synaptic cleft since it is known that N/OFQ receptor desensitization occurs over a matter of minutes (Pei et al., 1997; Ma et al., 1997; Morikawa et al., 1998; Hawes et al., 1998).

Short-term synaptic depression is the activity-dependent reduction in synaptic transmission that occurs within several hundred milliseconds after the onset of activity (Zucker, 1989). This type of synaptic plasticity acts as a temporal filter of presynaptic action potential firing frequency in that neurotransmission at particular frequency ranges is depressed while other frequencies transmit information reliably (Fortune and Rose, 2001). Recent evidence suggests that intra-SCN synaptic neurotransmission can change postsynaptic firing from a regular to an irregular pattern (Kononenko and Dudek, 2004). In light of this, the rate of presynaptic spiking could be considered an important determinant of the neural coding that controls postsynaptic firing patterns in the SCN, and synaptic plasticity could reasonably be expected to modulate this coding. Due to the ubiquity of GABAergic synaptic transmission between SCN neurons and its possible role in coupling the individual circadian oscillators into a unified circadian rhythm, the hypothesis was developed that the strength of synaptic transmission is not constant over a range of firing frequencies and times of day. The implications of such a variation in synaptic strength bear upon the coding of temporal information transmitted at the intra-SCN synapse.

Another form of synaptic modulation is the retrograde inhibition of presynaptic neurotransmitter release. In this mechanism, a postsynaptic cell fires action potentials that lead to Ca^{2+} influx and a subsequent Ca^{2+}-dependent release of a retrograde

transmitter. Retrograde transmitters can be released from either the postsynaptic dendrite or soma and act on either the presynaptic axon terminals or soma to inhibit neurotransmitter release. The advantages of this mechanism over the more common circuit, involving inhibitory interneurons feeding back upon the presynaptic cell, are its rapidity and the specificity with which individual synapses can be modulated (Alger, 2002). In the SCN, this type of modulation is particularly intriguing, due to the fact that neurons are tightly packed and receive multiple synaptic inputs from neighboring neurons (Guldner, 1984; van den Pol, 1986). Retrograde signaling would allow cells to inhibit specific synaptic inputs during times of elevated firing frequency, as is seen during the daytime. Global depolarization of SCN neurons in a slice preparation and staining for release-ready vesicles has shown evidence of dense core vesicles in somatic and dendritic locations (Castel, Morris, and Belenky, 1996), thereby demonstrating the possibility of extra-synaptic retrograde inhibition. Through the inhibition of presynaptic neurotransmitter release, this is another mechanism that might impact postsynaptic firing patterns (Kononenko and Dudek, 2004). Based upon the finding of dendritic dense core vesicles in a subpopulation of SCN neurons and the possibility that the release of the neuromodulator contained within these vesicles can influence the synaptic inputs these cells receive from neighboring SCN neurons, I tested the hypothesis that a subpopulation of SCN neurons can retrogradely inhibit specific synapses.

The final mechanism of synaptic modulation that will be examined is through G-protein coupled receptors that can be located at either the presynaptic axon terminal or the postsynaptic membrane. In the hypothalamus, many G-protein coupled receptors are responsive to a wide variety of peptides, possibly owing in part to the weak blood-

brain barrier in certain areas, such as the arcuate nucleus and median eminence, as well as the function of certain blood-borne peptides that act as organismal signals of metabolic states. While these peptides signal over relatively long distances—from one part of the organism to the hypothalamus—neuropeptides also serve a role in modulating local fast synaptic neurotransmission throughout the hypothalamus and in the SCN. Peptide production—the transcription/translation, splicing—and transport—the vesicular packaging and precursor peptide cleavage—generally requires significant amounts of cellular energy. This long process, however, allows the cell temporal and quantal control over signaling-peptide production (de Wied, 1987). In the SCN, where modulation of synaptic transmission may be effective in directing phase shifts or other events only during limited portions of the 24-hour cycle, these attributes may be especially well suited for modulation of synaptic transmission.

Nociceptin / Orphanin FQ: Previous studies have shown that vasoactive intestinal polypeptide (VIP) is a positive modulator of GABAergic neurotransmission in the SCN, and in this work, I examine the possibility that nociceptin / orphanin FQ (N/OFQ) is a negative modulator of GABAergic neurotransmission. The search for additional opioid-like receptors had previously lead to the discovery of an orphan receptor with low affinity binding to known opioids (Bunzow et al., 1994). Subsequently, the 17-amino-acid long peptide N/OFQ was discovered as its endogenous ligand (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ has a wide distribution throughout the central nervous system and generally inhibits neuronal firing rate in the majority of brain regions in which the receptor is expressed, suggesting that N/OFQ might function as the "peptidergic analogue to GABA" (Heinricher, 2003).

The role of this peptide in these various systems is often to inhibit the activity of fast synaptic neurotransmission, and thus the larger peptide, N/OFQ, that presumably requires significant amounts of cellular energy and time to produce limits the action of the smaller molecules, such as GABA and glutamate at the synapse. N/OFQ has also been shown to play a prominent role in modulating the activity of neurons in other regions of the hypothalamus (Wagner et al., 1998; Slugg et al., 1999). Previous evidence has shown that the vast majority of neurons in the SCN respond to N/OFQ by an evoked K^+-current, a reduction in baseline Ca^{2+} levels; and while it does not shift the phase of the circadian rhythm, the peptide inhibits the glutamate-mediated light-induced phase shift (Allen et al., 1999). Other (μ, ∂, κ) opioid activity is relatively limited in the SCN (Cutler and Mason, 1995) compared to N/OFQ, making it the primary peptide of this family of receptors that is active in the SCN. The hypothesis tested in this work, based on the observation that N/OFQ acts as an inhibitory neuropeptide in the vast majority of SCN neurons, is that N/OFQ, modulates intra-SCN GABAergic neurotransmission by inhibiting GABA release.

Entrainment of the circadian system to environmental light inputs

Light is the major environmental cue that the SCN uses to synchronize its endogenous rhythm with that of the environment, and a direct projection from a subset of retinal ganglion cells is responsible for the transmission of this information (Moore et al., 1995; Abrahamson and Moore, 2001; Pickard et al., 2002; Berson et al., 2002). Lesioning of the optic nerve results in the inability to adjust the endogenous phase of the SCN to light stimuli (Johnson et al., 1988). Light information is conveyed to the SCN from the retina via the retinohypothalamic tract (RHT), which terminates on SCN

neurons (Moore and Lenn, 1972; Groos and Meijer, 1985; Meijer et al., 1986). RHT inputs are excitatory and release the neurotransmitter, glutamate, which is found in both retinal ganglion cells and their axon terminals in the SCN (Moffett et al., 1990; van den Pol, 1991). Glutamate levels increase in the SCN after RHT stimulation and antagonists to glutamate receptors block the RHT input into the SCN (Liou et al., 1986; Shibata et al., 1986). The post-synaptic glutamate receptors AMPA and NMDA are found in the SCN, and specific agonists to these receptors mimic the effects of light on free-running circadian rhythms, while antagonists block light-induced phase-shifts (Cahill and Menaker, 1989; Ohi et al., 1991; Kim and Dudek, 1991; Colwell and Menaker, 1992; Shibata et al., 1994b). The activity of glutamate ultimately leads to postsynaptic Ca^{2+} influx that sets in motion a cascade of signal transduction events, culminating in the phosphorylation of the transactivating factor cAMP-response element binding protein (CREB) (Ding et al., 1997; McNulty et al., 1998; Schurov et al., 1999), which is required to phase-shift the circadian timing system. Thus, the specific innervation from the retina sets into motion a cascade of postsynaptic signal transduction events in the SCN that can shift the phase of circadian rhythms in mammals.

The first evidence that a unique set of retinal cells exist that might be responsible for the transmission of light information relevant to the circadian timing system came from the rodless / coneless mice. These mice lack both retinal rods and cones and are, therefore, unresponsive to normal visual stimuli. Yet, light entrainment of circadian activity rhythms and the acute light inhibition of melatonin secretion are maintained in these mice as long as the retinas are not enucleated (Freedman et al., 1999; Lucas et al., 1999). A novel photopigment in retinal ganglion cells, called melanopsin (Provencio et al., 1998), has since been confirmed as the primary photoreceptor responsible for light

entrainment of circadian rhythms (Gooley et al., 2001; Hannibal et al., 2002; Berson, Dunn, and Takao, 2002). Retinal cells projecting to the SCN fire transiently in response to light-induced depolarization and the light-induced depolarization, in these cells peaks after 5 sec of constant exposure (Warren et al., 2003). These data were obtained from retinas in which the synaptic inputs from rods and cones was blocked, so it is as yet unclear if the firing rates and trains of action potentials of these ganglion cells could be greater under normal conditions of intact rod and cone input. However, if synaptic transmission at the RHT terminal occurs primarily as a result of melanopsin activation, and these ganglion cells are quiescent under further light exposure during the remainder of the light period—something that has also not yet been examined—relevant modulation of the glutamatergic RHT inputs might occur only in a temporally limited time frame. In other words, though a modulator at the RHT-SCN synapse might be released at any time during the circadian cycle, it would be expected to affect the light input only during times of neurotransmitter release, which in the case of this population of ganglion cells, could turn out to be temporally very limited.

Presynaptic modulation of entrainment

Two examples of presynaptic modulators at RHT terminals are serotonin (5-HT) and GABA. Serotonin has been shown to inhibit light-induced phase shifts and c-fos expression (Pickard et al., 1996) via the presynaptic inhibition of glutamate-mediated currents (Pickard et al., 1999; Jiang et al., 2000). Similarly, GABA acting via the $GABA_B$ receptor, inhibits light-induced phase shifts (Ralph and Menaker, 1989), an action shown to have a presynaptic site of action (Jiang et al., 1995). While a projection from the median raphe is responsible for the release of serotonin in the SCN (Meyer-

Bernstein and Morin, 1996), a projection from the intergeniculate nucleus is responsible for release of GABA and neuropeptide Y (NPY) (Moore and Speh, 1993; Morin and Blanchard, 2001). Whether GABA released from this projection is, in turn, responsible for the observed presynaptic RHT inhibition (Lall and Biello, 2003), or whether postsynaptic SCN neurons are the primary source of this inhibitory message—or both—is unclear. The co-transported peptide, NPY, however, has been shown to inhibit glutamate release as well (van den Pol et al., 1996). Either way, these results make clear that afferents from multiple brain regions can modulate the retinal light inputs into the SCN with either peptide or non-peptide neuromodulators.

The events leading to neurotransmitter release and the subsequent postsynaptic response are often subject to regulation by neuromodulators. Presynaptic action potentials invade the axon terminals leading to Ca^{2+} influx through voltage-activated channels, this, in turn, results in neurotransmitter release and the neurotransmitter then activating postsynaptic receptors. In a sense, this final step is an amplification of the signal, since the neurotransmitter is acting on multiple postsynaptic receptors. An inhibitory neuromodulator acting postsynaptically could conceivably be overcome by an increase in presynaptic stimulation; whereas, a neuromodulator acting at multiple presynaptic sites may be a more effective inhibitor of neurotransmission. Therefore, determining the exact presynaptic modulatory mechanisms is an important piece of information to illustrate a neuromodulator's impact on synaptic transmission. An interesting example of this is demonstrated in the lateral geniculate, another retinorecipient area. Here, as in the SCN, both 5-HT and GABA demonstrate postsynaptic inhibitory actions, yet a strong inhibition of neurotransmission has been

shown to be mediated by a reduction in presynaptic Ca^{2+} levels (Chen and Regehr, 2003).

The inhibitory modulator at RHT terminals that is examined in this work is the 17-amino acid long neuropeptide nociceptin / orphanin FQ (N/OFQ), which has been mentioned briefly above. Previous evidence from our laboratory shows that the light-induced phase advance is inhibited by microinjection of this peptide (Allen, et al., 1999). N/OFQ receptor (ORL1) expression has been demonstrated in the retina (Makman and Dvorkin, 1997) and so, it is at least possible that the retinal ganglion cells projecting to the SCN could express this receptor at their axon terminals. Together, these results lead to the hypothesis that N/OFQ inhibits presynaptic glutamate release from RHT terminals in the SCN.

Specific Aims

Synaptic transmission in the SCN forms an integral part of the coupling mechanisms underlying interneuronal synchronization between the individual circadian oscillators of the SCN and also transmits relevant environmental information to the circadian system. The presynaptic release of GABA and glutamate acting on postsynaptic $GABA_A$, AMPA, and NMDA receptors in the SCN is a fast process capable of influencing postsynaptic membrane voltages for a matter of milliseconds or seconds. However, the compound effects of thousands of such synaptic events over the course of the 24-hour cycle may have a large impact on circadian physiology. The overriding hypothesis of this work has been that in order for fast synaptic transmission to convey relevant circadian—on the order of hours—information between SCN neurons, modulatory mechanisms refine this synaptic transmission. Therefore, the

following three specific aims were put forth to investigate the types of modulatory mechanisms that influence synaptic transmission in the SCN.

i. To determine whether GABA-mediated currents evoked at relevant firing frequencies display synaptic plasticity. While it has been known for some time that a majority of cells in the SCN display a circadian rhythm in action potential firing frequency that peaks during the subjective day and that these cells form GABAergic synapses between neighboring cells, it is unknown whether synaptic strength remains constant over the entire range of possible firing frequencies. Synaptic plasticity is a common and well-characterized mechanism of coding relevant information between cells throughout the central nervous system. Synaptic plasticity at intra-SCN synapses can indicate significant filtering (as shown in paired-pulse depression) or amplification (as shown in paired-pulse facilitation) of information transmitted at certain frequencies. A diurnal rhythm in the magnitude or direction of synaptic plasticity could indicate that this phenomenon is under the direct control of the circadian clock.

The hypothesis that synaptic plasticity exists at frequencies relevant to SCN physiology was tested by the use of paired evoked $GABA_A$-mediated currents. A highly significant correlation between the time of day and the degree of paired-pulse depression was found in the study described in Manuscript #1 of the Results section. A partial examination of the mechanism responsible for this and its implications are discussed.

ii. Retrograde inhibition at intra-SCN GABA synapses. The endogenous circadian rhythm expressed in the majority of SCN neurons demonstrates that neuronal excitability is greater during the subjective day than the subjective night. During this time of heightened excitability, an SCN cell could encounter an increase in inhibitory

input from neighboring SCN cells that are also exhibiting greater firing frequencies. GABAergic input has been shown to affect postsynaptic firing patterns (Kononenko and Dudek, 2004), yet it is unclear whether a cell can overcome this influence. Therefore, the specific aim of Manuscript #2 is to examine whether a postsynaptic cell can directly silence specific synaptic inputs via retrograde inhibition.

The hypothesis that postsynaptic depolarizations evoked at a physiologically relevant frequency can inhibit presynaptic GABA release was tested using evoked $GABA_A$-mediated currents. It was found (Manuscript #2) that a subset of SCN neurons can inhibit specific GABAergic inputs in a Ca^{2+} - dependent process mediated by a presynaptic pertussis-toxin-sensitive G-protein coupled receptor. A discussion of possible mechanisms and the implications of these results are presented.

iii. Nociceptin / Orphanin FQ is an important inhibitory neuromodulator at both GABA and Glutamate synapses in the SCN. GABA- and glutamate-mediated currents encompass the majority of the fast electrochemical communication in the local circuitry within the SCN (Jiang et al., 1997a). It is known, however, that particular neuropeptides enhance the strength of these synaptic inputs. For instance, pituitary adenylate cyclase activating protein (PACAP) enhances glutamatergic neurotransmission and VIP enhances GABAergic neurotransmission in the SCN. Following previous results (Allen et al., 1999) that showed N/OFQ evoked a K^+-current in the vast majority (88%) of SCN neurons and inhibited the light-induced phase advance, the hypothesis was advanced that this peptide inhibits both glutamatergic, as well as GABAergic, release in the SCN.

Examination of evoked currents showed that N/OFQ inhibited both GABAergic and glutamatergic current amplitudes in a dose-dependent fashion at a presynaptic site

of action. Manuscript #3 discusses the presynaptic mechanisms of N/OFQ action at RHT terminals.

iv. Major conclusions. Several major conclusions arise from these studies. For one, intra-SCN synaptic communication is highly regulated, and the degree of this regulation is dependent upon the time of day. Specifically, synaptic plasticity exhibits a diurnal variation and retrograde inhibition, a mechanism by which postsynaptic cells can inhibit synaptic input, depends upon the action potential firing frequency that is regulated by the circadian clock. Also, the effects of the inhibitory peptide N/OFQ are widespread on both excitatory and inhibitory synaptic neurotransmission. While other peptides are known to modulate synaptic neurotransmission in the SCN, no inhibitory neuropeptide has thus far been shown to exert this degree of influence.

Results

Manuscript 1

GABAergic synapses of the suprachiasmatic nucleus exhibit a diurnal rhythm of short-term synaptic plasticity

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Abstract:

We examined synaptic plasticity at intra-suprachiasmatic nucleus (SCN) GABAergic synapses by measuring the paired-pulse ratio between pairs of evoked inhibitory postsynaptic currents (IPSCs). Inter-stimulus intervals were chosen to represent the range of spontaneous action potential firing frequencies found in SCN neurons. A majority of synapses studied during the day exhibited paired-pulse depression (PPD), while a majority of synapses studied during the night showed no PPD. Two types of PPD were found. Type 1 PPD expresses the greatest inhibition at shorter inter-stimulus intervals, is predominant in the early morning and is likely due to vesicle depletion. Type 2 showed the greatest inhibition at inter-stimulus intervals between 175-225 msec, is found throughout the day yet rarely at night and is likely due to a Ca^{2+}-dependent mechanism that is independent of pertussis toxin sensitive G-proteins. Thus, multiple mechanisms of synaptic plasticity modulate intra-SCN communication throughout the diurnal cycle.

Introduction:

The mammalian hypothalamic suprachiasmatic nuclei (SCN) are the site of an endogenous, self-sustained circadian timekeeper that provides temporal information for a wide range of physiological processes and behaviors. The circadian rise and fall of excitability of SCN neurons results in a circadian rhythm of peak ensemble action potential firing frequency which occurs during the middle of the day (Green and Gillette, 1982;Smith, Inouye, and Turek, 1989;Walsh, et al., 1992). Assuming that the action potentials recorded from the soma of SCN neurons are faithfully transmitted to

the axon terminals, we examine here whether synaptic efficacy remains constant over a range of observed SCN action potential firing frequencies.

The major neurotransmitter at intra-SCN synapses is GABA, and nearly all neurons in the SCN contain glutamic acid decarboxylase, the GABA synthesizing enzyme (Moore and Speh, 1993;Belenky et al., 2003). Postsynaptic $GABA_A$ receptors connect the local synaptic circuitry (Jiang et al., 1997a) and may contribute to the synchronization of SCN firing rate rhythms (Liu and Reppert, 2000). During the day, GABAergic SCN neurons fire action potentials at frequencies between 2 and 22 Hz, with an average frequency of 5 - 7 Hz (Groos and Hendriks, 1982;Welsh et al., 1995;Jiang et al., 1997a;Pennartz et al., 1998). We propose that this range of IPSC frequencies might be instrumental in information processing within the intra-SCN circuitry and that short-term changes of synaptic efficacy could refine this signaling.

Although GABAergic synapses are almost ubiquitous between SCN cells, it is unclear what role these synapses play in signaling a cell's relative circadian phase to neighboring cells within the circuit. GABA has different actions at different times of the day. For example bicuculline, a $GABA_A$ antagonist, blocks the phase delay of light pulses administered during the early night while it has no effect on phase advancing light pulses during the late night (Ralph and Menaker, 1985). Also, GABA may switch from an inhibitory to an excitatory neurotransmitter during certain times of the circadian cycle (Wagner et al., 1997), a finding that remains controversial (Gribkoff et al., 2003). While these studies address possible qualitative day-night differences in $GABA_A$-mediated neurotransmission, short-term synaptic plasticity, a common mechanism for modulating synaptic communication throughout the central nervous system (Zucker and Regehr, 2002), has not been examined at intra-SCN synapses.

Examining paired evoked IPSCs at different frequencies encompassing the range of the endogenous daytime spontaneous action potential firing rates (Groos and Hendriks, 1982;Welsh et al., 1995;Jiang et al., 1997a;Pennartz et al., 1998) we report a significant diurnal rhythm in synaptic plasticity. Intra-SCN synapses exhibit paired-pulse depression (PPD) at short inter-stimulus intervals during the early day yet not at other times of the day. A median range of inter-stimulus intervals, around the average range of daytime spontaneous firing frequencies of SCN neurons exhibits PPD mainly during the mid-day, but rarely at night. Our results demonstrate the existence of short-term synaptic plasticity in the SCN and imply a role for this mechanism in the integration of temporal information between SCN neurons.

Materials and Methods:

Preparation of brain slices

Male Sprague-Dawley rats (6-8 wk old) were maintained on a 12 hr light / 12 hr dark lighting schedule for at least one week. During the light phase, rats were deeply anesthetized with halothane, their brains removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 20, $MgCl_2$ 3.6, $CaCl_2$ 0.5, and glucose 11 saturated with 95% O_2 / 5% CO_2. The Institutional Animal Care and Use Committee of OHSU approved all experimental procedures involving animals and all efforts were made to minimize pain and the numbers of animals used.

Coronal (250 μm thick) hypothalamic slices containing the SCN were prepared on a vibrating blade microtome (Leica VT1000S, Nussloch, Germany) and transferred to the recording chamber where they were completely submerged in 32°C ACSF (same

as above, except $MgCl_2$ 1.2, $CaCl_2$ 2.4 mM). Experiments were performed with the SCN visualized using infrared differential interference microscopy (IR-DIC). A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) attached to a stimulus isolation unit (Grass Instruments, Quincy, MA) was placed into the lateral SCN, approximately 100 – 200 μm dorsal and lateral to the recording site in the ventromedial SCN. The stimulus amplitude (20 – 50 V) was set to the minimum value that reliably evoked both the primary and secondary IPSC at ten different inter-stimulus intervals (25 - 475 msec). Each pair of stimuli was separated by 12-seconds to allow sufficient time for recovery from inhibition.

Patch clamp recording

Whole-cell patch clamp recordings were performed 0.5 to 10 hr after slice preparation in neurons located in the ventromedial portion of the SCN. Whole-cell patch electrodes had outside tip diameters of ~1 μm and resistances of ~5 MΩ when filled with a solution containing (in mM): $CsCH_3O_3S$ 87, CsCl 20, $CaCl_2$ 1, HEPES 10, EGTA 11, CsOH 25, ATP 3, GTP 0.3, QX-314 0.5, pH 7.4. The electrode was advanced into the brain slice and a seal with the cell membrane (resistance: 2-15 GΩ) was obtained by applying negative pressure. After further negative pressure to rupture the cell membrane, currents were measured with an Axopatch-1D amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz, and recorded at 10 kHz using the data acquisition program PulseFit (HEKA, Lambrecht, Germany) on a Macintosh. The holding potential for whole cell voltage clamp recordings was –60 mV, liquid junction potential was not corrected for. GABA-mediated currents were pharmacologically isolated by bath application of the selective AMPA receptor blocker 6-cyano-7-

nitroquinoxaline-2,3-dione (CNQX, 5 μM, Sigma, St. Louis MO) and the selective NMDA receptor blocker D(-)-2-Amino-5-phosphonopentanoic acid (D-APV, 50 μM, Sigma, St. Louis, MO). Recorded currents were confirmed to be $GABA_A$-mediated by blocking currents with picrotoxin (50 μM) after completion of selected experiments. Certain experiments were performed following a 20-minute preincubation with N-ethylmaleimide (NEM, 200 μM, Sigma, St. Louis, MO) or the selective $GABA_B$ receptor antagonist saclofen (100 μM, Sigma, St. Louis, MO).

Paired-pulse ratio recordings

Pairs of stimuli were applied to evoke inhibitory postsynaptic currents (IPSCs) at each of 10 inter-stimulus intervals between 25 – 475 msec and all 10 pairs were repeated at least three to six times for each recorded cell. Absolute current amplitudes of both the first and second IPSC (IPSC1, IPSC2) were measured from the current level immediately preceding the stimulus artifact to the maximal current amplitude. Current rundown was not a factor in these recordings in cells recorded for longer than 10 minutes; IPSC1 amplitudes at the beginning (103.7 ± 4.4 pA), and at the end of recording (99.1 ± 3.9 pA) were not significantly different ($p = 0.08$, $n = 49$).

Data analysis

At each inter-stimulus interval, multiple (3 – 6) current amplitudes of the first IPSC (IPSC1) and the second IPSC (IPSC2) were measured for every cell recorded. To calculate paired-pulse ratios for each inter-stimulus interval, the mean amplitude of all IPSC2 was then divided by the mean amplitude of all IPSC1. Current amplitudes were averaged to obtain the mean paired-pulse ratio instead of averaging the paired-

pulse ratios of each individual trial to avoid false interpretations of paired-pulse facilitation (Kim and Alger, 2001). Current pairs were not included into the averaged results when either the first or the second pulse failed to evoke an IPSC. Failures were determined visually when the current was not different from baseline at the expected time interval after the stimulus artifact (1 - 3 msec). Current pairs were also excluded when either the first or the second evoked current had a spontaneous current superimposed upon it.

After current responses were classified according to their paired-pulse ratio, we examined whether individual animal variability could be the cause of the apparent circadian variation in response types. Of the slices from which cells were recorded during both the early daytime hours as well as the late daytime hours, 82% (9 of 11) showed both the early daytime phenotype (Type 1, see results below) as well as the later phenotype (Type 2 or no inhibition, see results below). This makes unlikely the possibility that cells from individual animals recorded primarily during the early or late day might make the results appear to follow a diurnal rhythm.

Statistical Analysis

After observing three types of paired-pulse responses over the range of inter-stimulus interval recorded (Type 1, 2, and no PPD, see below), statistical analyses were performed using SYSTAT (SPSS, Evanston, IL). At each inter-stimulus interval, statistically significant differences between response types were determined using repeated-measures ANOVA. Individual comparisons between groups at each inter-stimulus interval were then tested using the Bonferoni correction.

To study the diurnal rhythm of PPD, a log-linear model was fitted to the number of cells exhibiting Type 1, Type 2 or no PPD for each one-hour interval of projected time (Christensen, 1997; Lindsey, 1995). These models extend regression analysis by accommodating frequency or count data, which because of their discrete nature, are not normally distributed. Specifically, the number of events recorded over a fixed interval of time is presumed to follow a Poisson distribution for which the mean number of events depends on other measured variables (projected time in these experiments). The log-linear model uses a multi-way frequency table (crosstabulation table) to analyze the interactions between each factor in the table for statistical significance (Lindsey, 1995; Christensen, 1997).

Results:

Paired-pulse ratio

Paired inhibitory postsynaptic currents (IPSCs) were recorded in response to electrical stimulation of the SCN at inter-stimulus intervals of 25 - 475 msec. These inter-stimulus intervals correspond to frequencies of 40 - 2.1 Hz, which is similar to the previously reported range of spontaneous daytime action potential firing frequencies of 0.12 – 22 Hz (Groos and Hendriks, 1982;Welsh et al., 1995;Jiang et al., 1997a;Pennartz et al., 1998).

Of a total of 63 cells examined at inter-stimulus intervals between 25 and 475 msec, the paired-pulse ratio of 29 cells exhibited repeatable paired-pulse depression (PPD, defined as IPSC2 < IPSC1). This PPD occurred most frequently at inter-stimulus intervals between 25 and 275 msec. (25 msec: 0.67 ± 0.05; 275 msec: 0.77 ± 0.03, Fig. 1B), while inter-stimulus intervals from 325 – 475 msec rarely showed a

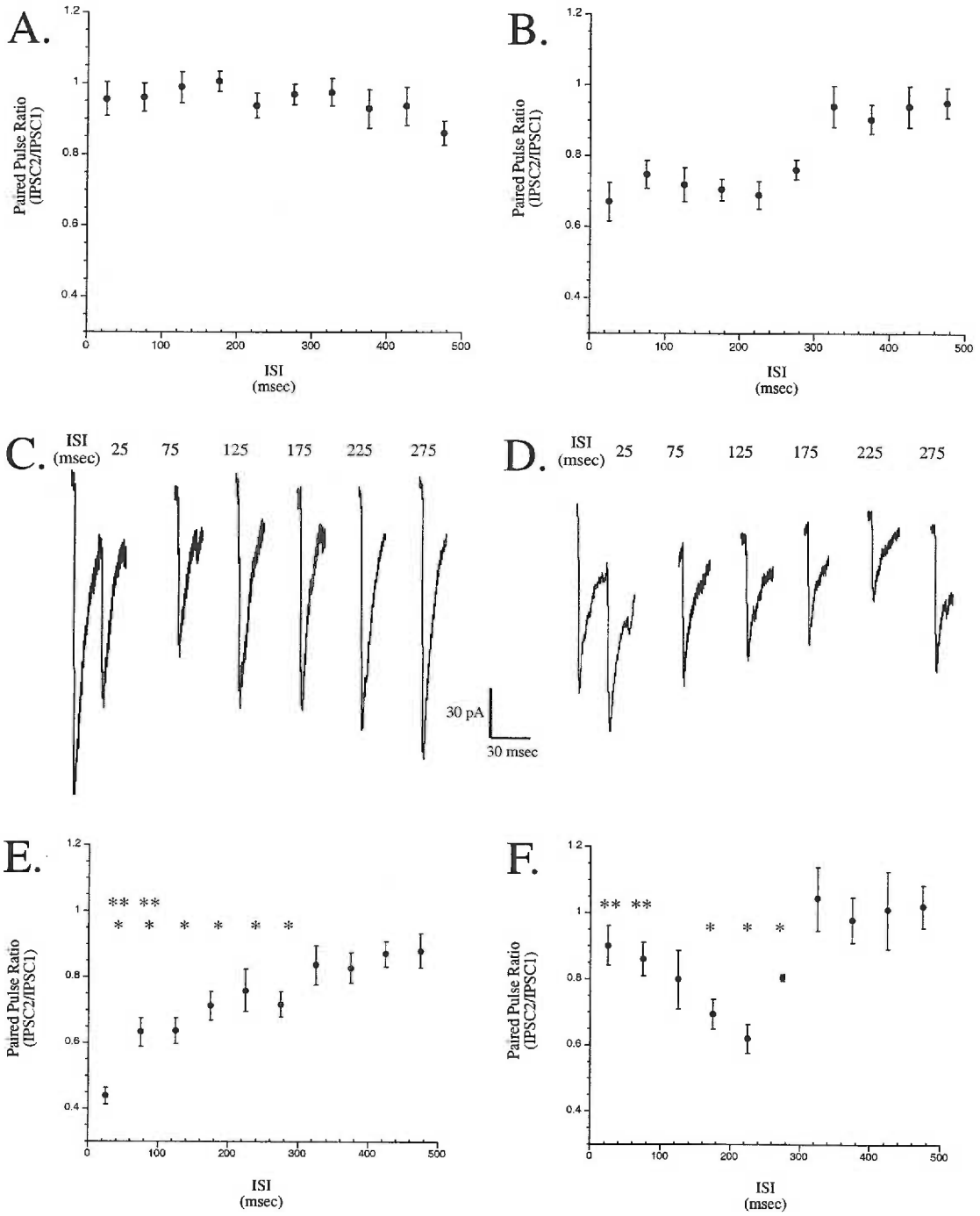

Figure 1. Current amplitude ratios from paired evoked IPSC measurements. A, B, E, F: For each inter-stimulus interval, all amplitudes for the first IPSC (IPSC1) and the second IPSC (IPSC2) for each cell were averaged and expressed as the paired-pulse ratio (IPSC2 / IPSC1). A. Paired-pulse ratios of cells not exhibiting PPD (n = 34). B. Averaged paired-pulse ratios of all cells exhibiting mean paired-pulse depression at any inter-stimulus interval (n = 29). C. Example currents (average of 3 sweeps) from a recording exhibiting Type 1 PPD. D. Example trace (average of 3 sweeps) from a recording exhibiting Type 2 PPD at inter-stimulus intervals between 175 and 225 msec. E. Average paired-pulse ratios of all cells showing Type 1 PPD (* = significantly different from synapses exhibiting no paired-pulse depression (A), $p < 0.05$, ** = significantly different between Type 1 and Type 2 (F), $p < 0.01$, n = 16, repeated measures ANOVA). F. Average paired-pulse ratios of all cells showing a Type 2 pattern of PPD (* = significantly different from synapses exhibiting no paired-pulse depression (A), all points are mean ± S.E.M. n = 13, $p < 0.005$, repeated measures ANOVA).

paired-pulse ratio of < 1. The remaining (34) cells showed no apparent PPD, and ratios at all inter-stimulus intervals did not differ from one another (Fig. 1A, right; $p > 0.4$, repeated measured ANOVA).

While recording paired currents, it was observed that some synapses exhibited the greatest PPD at shorter inter-stimulus intervals and others showed the greatest depression at inter-stimulus intervals between 175 – 275 msec. Accordingly, the variability in PPD was considerably greater at shorter inter-stimulus intervals—the coefficient of variation (CoV) at 25 msec was 0.43—than at longer inter-stimulus intervals (CoV = 0.23 at 175 msec). Since this suggests multiple PPD types, we separated the PPD responses into two types according to the inter-stimulus interval evoking the largest PPD. One group of paired IPSCs ("Type 1", Fig. 1C, E, n = 16) exhibited an inverse relationship between the magnitude of inhibition and inter-stimulus interval, and was seen primarily during the early day (Zeitgeber time (ZT) 0 – 4, see below). In the Type 1 PPD, the greatest amount of depression (lowest paired-pulse ratio) was 0.44 ± 0.02 at the 25 msec inter-stimulus (CoV = 0. 23). In cells showing Type 1 PPD, the PPD at inter-stimulus intervals between 25 and 275 were significantly different ($p<0.05$, ANOVA, Bonferoni post-hoc) from the paired-pulse ratio in the neurons that did not display PPD. The timecourse at which ratios approached values of 1:1 fit a single exponential ($R^2 = 0.93$) with $\tau = 173$ msec.

The second response type ("Type 2", Fig. 1D, F, n = 13), exhibited a maximal PPD to 0.62 ± 0.04 of control (CoV = 26) at 225 msec, with ratios closer to 1 at shorter (25 msec: 0.9 ± 0.05), and longer (325 msec: 1.04 ± 0.09) inter-stimulus intervals. Ratios of this response type were significantly different from the "no PPD" type at inter-stimulus intervals of 175, 225 and 275 msec ($p<0.005$, ANOVA Bonferoni). Type

2 responses were seen throughout the daytime hours (ZT 4 – 8, see below). Type 1 and 2 PPD were significantly different at the shorter inter-stimulus intervals (25 and 75 msec, $p<0.01$, ANOVA Bonferoni), but at no other inter-stimulus interval.

While Type 1 synapses displayed strong PPD at shorter inter-stimulus intervals, the PPD at the medium range (175 – 225 msec) was not significantly different from Type 2 cells. Also, the timecourse at which ratios approached 1:1 in Type 1 cells (τ = 173 msec) was long enough to allow for the possibility that Type 1 cells express two mechanisms of PPD while Type 2 synapses express only the one that leads to PPD at medium-range inter-stimulus intervals. To examine possible mechanisms contributing to PPD, we further examined PPD at inter-stimulus intervals of 25 and 200 msec.

Circadian Rhythm of PPD

A clear diurnal rhythm was found in the times at which Type 1, Type 2, and non-PPD responses could be observed. All Type 1 PPD recordings occurred from ZT 20 – 5.5. Moreover, the majority (83%) of PPD between ZT 0 – 4 exhibited a Type 1 pattern (Fig. 2A). Type 2 PPD occurred over a much wider span of the circadian cycle, yet were most frequently observed (77%) during the day (ZT 0 – 12, Fig. 2A). Most Type 2 responses clustered around the middle of the day; 46% of cells recorded between ZT 4 – 8. While the evoked currents which showed no PPD could be observed throughout the day (33%), the overwhelming majority of cells (82%) recorded during the night (ZT 12-24, Fig. 2A) showed no PPD. This indicates that the likelihood of recording a particular type of PPD in the SCN depends upon the time of recording.

To describe this, the PPD type as a function of Zeitgeber time was assessed using a probability model. Raw data of the counts of each type of PPD were fitted to a

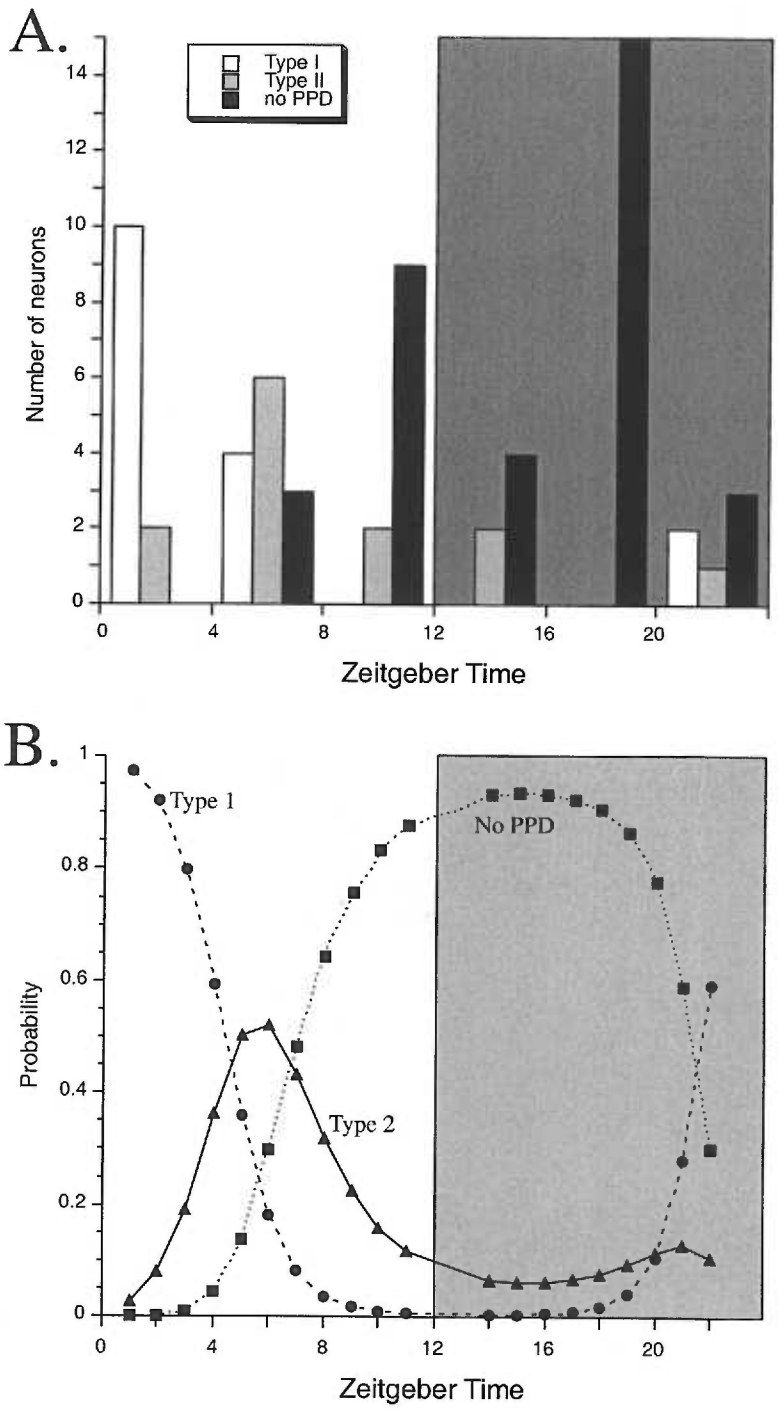

Figure 2. Diurnal rhythm of PPD in the SCN. A. Total number of neurons of Type 1 (open bars, n = 16), Type 2, (shaded bars, n = 13), and no PPD (black bars, n = 34) for each four hour bin of the 24 hour day. B. Plot of the log-linear model fitting the number of synapses exhibiting Type 1 (circles), Type 2 (triangles), or no PPD (squares) for each one-hour bin. Over 24 hours, the probability that any given synapse exhibits a particular response type strongly varies, ($X^2_{34} = 27.25$, $p = 0.787$; Deviance goodness-of-fit test) such that Type 1 responses are most likely found during the late night (ZT 20) to early morning (ZT 4), Type 2 during the mid-day (ZT 2-10) and no PPD responses are found primarily at night.

probability function on a continuous 24-hour cycle. Figure 2B depicts the probabilities that a given cell exhibits either Type 1, Type 2, or no PPD for each 1-hour bin of the 24-hour cycle. The values and resulting curves are derived from a log-linear model fitted to the number of synapses exhibiting each type of PPD (1, 2, or none) for each one-hour bin during the 24-hour cycle (Fig. 2B). To account for the curvature of the probability function of PPD Type vs ZT, the model has to match the number of actually recorded cells. Accordingly, the final log-linear model showed no evidence of inadequacy (X^2_{34} = 27.25, p = 0.787; Deviance goodness-of-fit test). Over a 24-hour cycle, the predicted value divided by the sum total of observed types describes the pattern of change of PPD type over time. According to this model, Type 1, Type 2, or no PPD clearly differed according to type.

After observing that Types 1 and 2 were found primarily during the early day, we performed experiments to rule out the possibility that these responses run down during the day while the slices are maintained *ex vivo*. Cells recorded from slices prepared from three animals sacrificed at ZT 8.5 did not exhibit either Type 1 or Type 2 PPD when recorded from ZT 9.5-12 (n = 6). The results from cells recorded from these slices were not different from cells recorded from slices prepared earlier in the day (ZT 0-2).

Vesicle depletion hypothesis

We examined whether separate mechanisms may be responsible for the PPD observed at short (25 msec) and at longer (200 msec) inter-stimulus intervals. In other systems, a frequently observed mechanism of PPD is the release-dependent depletion of the vesicle pool (Debanne et al., 1996). We tested whether either the PPD at 25 msec or

the PPD at 200 msec, or both may be due to vesicle depletion. One characteristic feature of vesicle depletion is that, when IPSC1 amplitudes are greater than average, IPSC2 amplitudes tend to be smaller than average. In other words, while IPSC amplitudes may vary from trial to trial in any cell, the vesicle depletion model predicts that the relative depression of IPSC2 amplitude depends upon the relative amplitude of IPSC1 (Zucker, 1989;Thomson, 2000). Experiments shown in Fig. 3 examine this possibility on 86 pairs of IPSCs from 25 Type 1 cells that showed PPD at 25 msec (ZT 3 - 5, Fig. 3A) and 50 IPSC pairs from 15 Type 2 cells (ZT 3 - 8, Fig. 3B). Individual IPSC1 and IPSC2 amplitudes from each pair of currents were normalized to the mean current amplitude of all the IPSC1 amplitudes recorded from that cell. A negative slope—as shown in Figure 3A (slope = -0.23, $p = 0.05$)—indicated a dependence of IPSC2 on the amplitude of IPSC1, and hence supported the possibility that the PPD at 25 msec inter-stimulus interval may be due to vesicle depletion (Zucker, 1989). A non-negative slope—as shown in Fig. 3B (slope = 0.51, $p = 0.0002$)—indicated independence of the amplitude of IPSC2 from the amplitude of IPSC1, and therefore an important prediction of the vesicle depletion model was not met for Type 2 PPD (Kraushaar and Jonas, 2000). If the PPD observed at both shorter (25 msec) and longer (200 msec) inter-stimulus intervals were caused by the same mechanism of action, both slopes would have been either negative or non-negative. This was not the case, however, indicating that the two types of PPD observed in the SCN are mechanistically distinct.

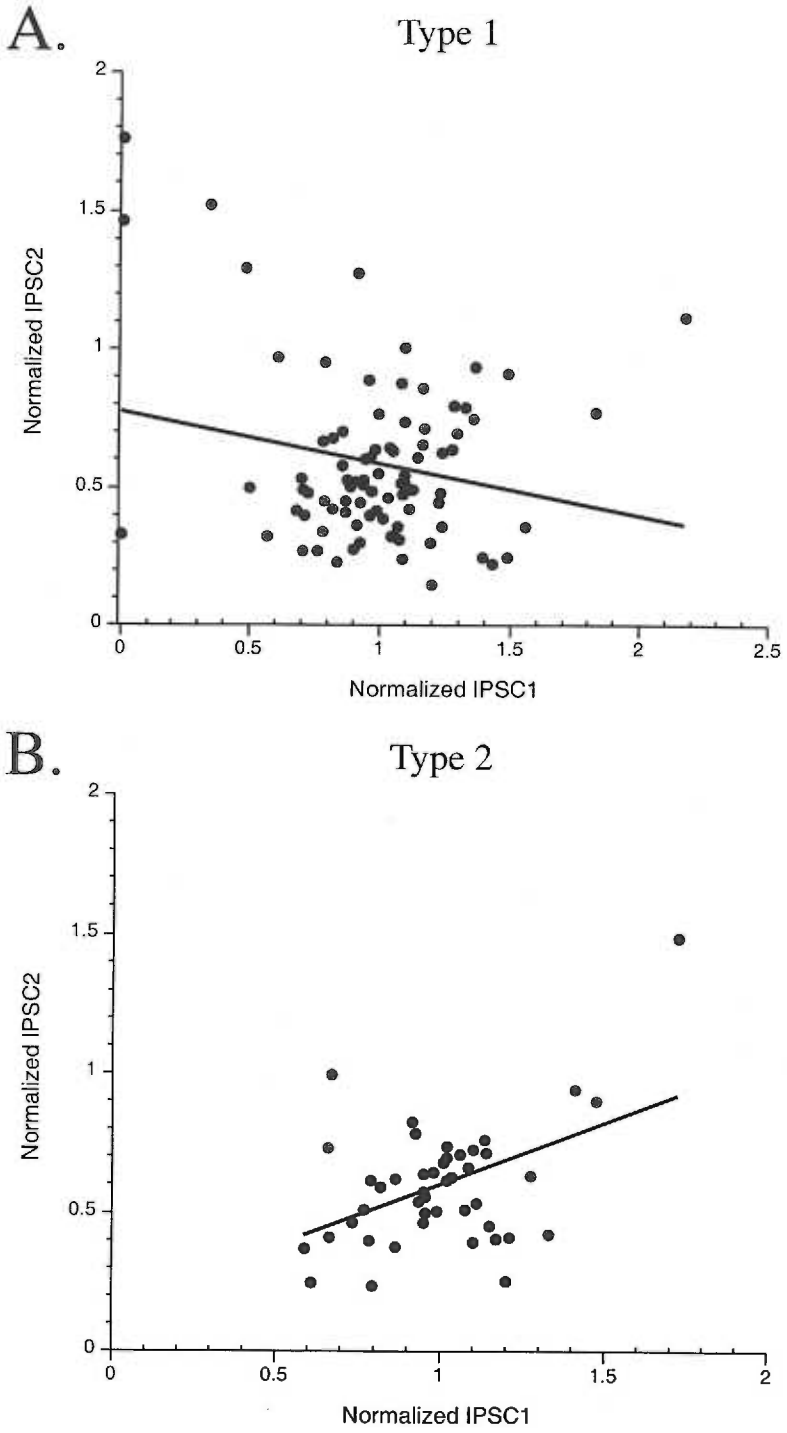

Figure 3. Separate mechanisms are responsible for Type 1 and Type 2 PPD. Pairs of currents (n = 86) were examined from synapses exhibiting Type 1 PPD at 25 msec inter-stimulus interval (Fig. 3A, n = 25), and pairs (n = 50) exhibiting Type 2 PPD at 200 msec inter-stimulus interval (Fig. 3B, n = 15). Each current amplitude was normalized to the mean current amplitudes for all IPSC1 in that recording. A: Scatter histogram of normalized pairs of currents exhibiting Type 1 PPD. Slope = -0.23, p = 0.05. B: Scatter histogram of normalized pairs of currents exhibiting Type 2 PPD. Slope = 0.51, p = 0.0002. The data indicate that the PPD observed at 25 msec inter-stimulus interval was likely due to neurotransmitter vesicle depletion.

PPD at 200 msec inter-stimulus interval

To determine whether repeated $GABA_A$ receptor activation leads to inhibition of current amplitude at this frequency, we used the $GABA_A$ agonist muscimol. Current amplitudes in response to paired (200 msec) focal application of muscimol (200 μM, 1 msec application time) were measured in five cells recorded between ZT 3 – 8. The average paired ratio for these currents was 1.00 ± 0.04. If receptor desensitization due to repetitive stimulation (Jones and Westbrook, 1995) were responsible for the PPD observed at 200 msec, we would have expected at least 77% of these neurons to exhibit a smaller response to the second muscimol application at these circadian times.

Release probability at 200 msec inter-stimulus interval

We examined whether neurotransmitter release probability might play a role in the observed PPD at the 200 msec inter-stimulus interval by calculating the failure rates of the first vs. the second IPSC. Failures were rare and determined visually when no change in baseline current could be detected at the time intervals at which evoked IPSCs were expected (1-3 msec after the end of the stimulus artifact). The failure rate ($n = 330$ current pairs from 22 cells, 200 msec inter-stimulus interval) for IPSC2 (4.7 %) was significantly greater than the failure rate for IPSC1 (1%, $p<0.01$, paired t-test). The differences in failure rates indicated that the probability of release was greater for IPSC1 than IPSC2 in each pair of IPSCs. Taken together, this suggests that at least part of mechanism responsible for the Type 2 PPD in the SCN is presynaptic (Jiang et al., 2000).

Next we examined whether experimentally lowering release probability for IPSC1 can change the PPD (200 msec inter-stimulus interval). The Ca^{2+} / Mg^{2+} ratio

was varied from 2 (2.4 mM Ca^{2+} / 1.2 mM Mg^{2+}) to 35 (3.5 mM Ca^{2+} / 0.1 mM Mg^{2+}) and 0.16 (0.5 mM Ca^{2+}/ 3.1 mM Mg^{2+}) in cells (n = 8, ZT 2-5) exhibiting PPD at 200 msec inter-stimulus interval. Currents evoked in standard and low Ca^{2+} solutions are depicted in Fig. 4A. As seen in Fig. 4B, raising the Ca^{2+}/Mg^{2+} ratio to 35 made no difference in the recorded paired-pulse ratio, however, lowering it to 0.16 abolished PPD. While both IPSC1 and IPSC2 amplitudes were reduced in the lowered Ca^{2+} solution (Fig. 4A), the reduced PPD in lowered Ca^{2+} is correlated (Fig. 4C) with a reduction in IPSC1 amplitude, indicating that reducing the release probability of IPSC1 also reduces PPD. Currents that did not exhibit PPD in the standard extracellular solution also did not exhibit PPD in either the increased or decreased Ca^{2+} solution. Therefore, the release probability during IPSC1 can be seen as high relative to the release probability during IPSC2.

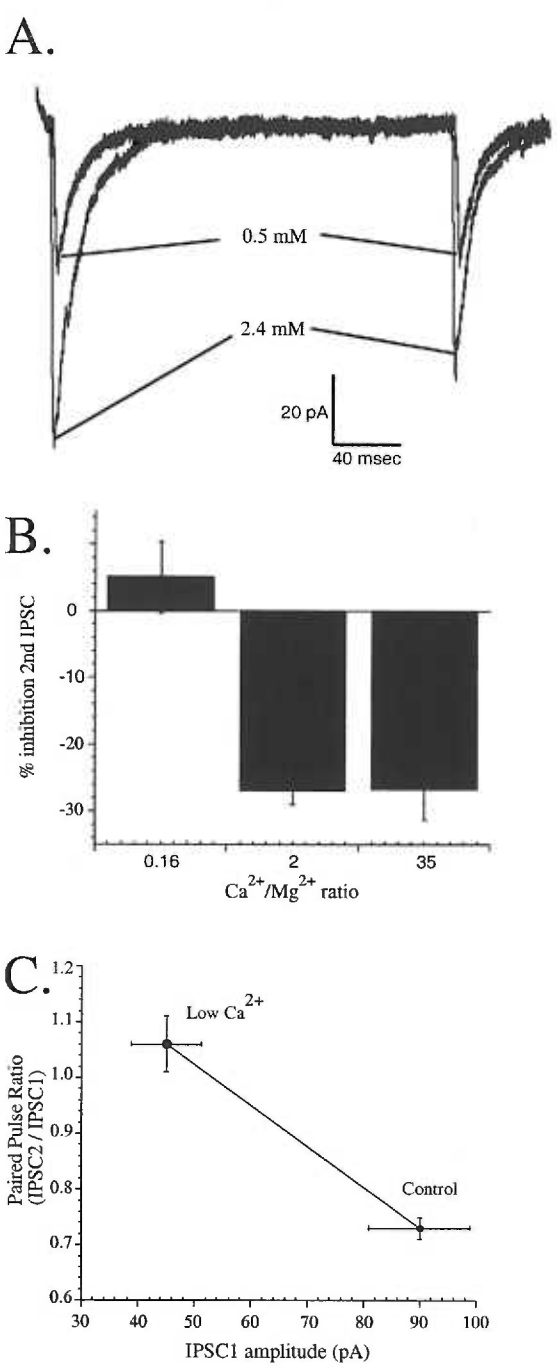

Figure 4. Paired-pulse ratios at varying Ca^{2+} / Mg^{2+} ratios. Regular extracellular solution contains 2.4 mM Ca^{2+} and 1.2 mM Mg^{2+} (ratio of 2), a ratio of 0.16 corresponds to 0.5 mM Ca^{2+} and 3.1 mM Mg^{2+}, and a ratio of 35 corresponds to 3.5 mM Ca^{2+} and 0.1 mM Mg^{2+}. A. Paired currents in regular (2.4 mM) and lowered (0.5 mM) Ca^{2+} . Note that while IPSC1 and IPSC2 are closer in amplitude (i.e. no PPD) in lowered Ca^{2+} extracellular solution, the absolute amplitudes of both are smaller than in the regular solution. B. Averaged (n = 9 each) paired-pulse ratios (200 msec inter-stimulus interval) of synapses recorded from ZT 2-6. Paired-pulse ratios approached 1:1 in the 0.5 mM Ca^{2+} solution while the ratio was not changed from baseline in the 3.5 mM Ca^{2+} solution. C. Average effect of low Ca^{2+} on IPSC amplitude and PPD (IPSC2 / IPSC1). Decreased release produced a parallel reduction in EPSC amplitude and PPD.

G-protein coupled receptor involvement in PPD

Presynaptic G-protein coupled receptor inhibition could act as a high-pass filter, reducing the amplitude of low-frequency signals while reliably transmitting high-frequency signals (Bertram, 2001). One possible mechanism for PPD is GABA acting on $GABA_B$ autoreceptors to decrease GABA release. Twelve cells exhibiting paired-pulse depression at the 200 msec inter-stimulus interval (5 Type 1, 7 Type 2 PPD) between ZT 3-8 showed no difference in PPD in the presence of 100 μM saclofen (Fig. 5A), a selective $GABA_B$ receptor antagonist.

However, other neuromodulators are expressed in the SCN that could be co-released with GABA. Possible modulators could act on pertussis-toxin sensitive G-protein coupled receptors expressed in the SCN. To determine whether other inhibitory G-protein coupled receptor mechanisms might be responsible for PPD, we examined the effect of 200 μM N-ethylmaleimide (NEM), an agent known to uncouple Gi and Go from G-protein coupled receptors (Asano and Ogasawara, 1986). After allowing a 20 min wash-in time, cells (n = 6) that exhibited PPD (3 Type 1, 3 Type 2) showed no significant difference in paired-pulse ratio (0.61 ± 0.06 control, vs. 0.66 ± 0.02 NEM, 200 msec inter-stimulus interval, Fig. 5B). Therefore, pertussis-toxin sensitive G-protein coupled receptor mechanisms appear unlikely to be involved in the PPD observed at intra-SCN synapses.

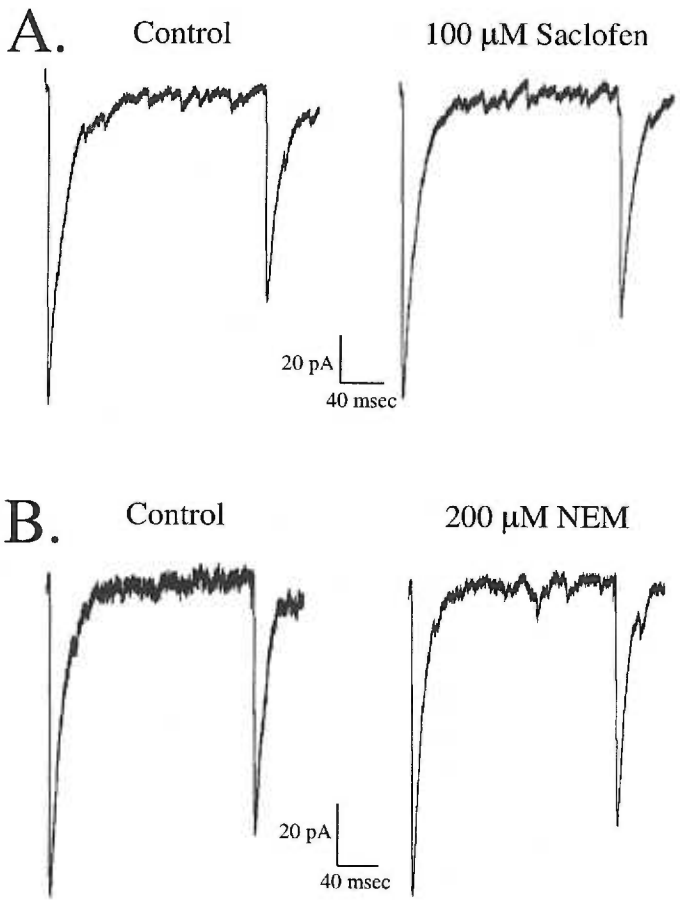

Figure 5. Lack of pertussis toxin-sensitive G-protein coupled receptor involvement in Type 2 PPD. Averaged traces from 5 paired IPSCs (each) from two Type 2 cells, showing that the magnitude of PPD at 200 msec inter-stimulus interval is unchanged from control in the presence of either 100 μM saclofen or 200 μM NEM. These data indicate that neither $GABA_B$ nor any other pertussis toxin-sensitive G-protein coupled receptor was involved in Type 2 PPD.

Discussion

The main finding of this study was a diurnal rhythm in short-term plasticity at intra-SCN GABAergic synapses. PPD at inter-stimulus intervals similar to the endogenous daytime action potential firing rate occurred most frequently during the daytime. PPD at higher stimulus frequencies occurred exclusively between ZT 20 - ZT 5.5. In contrast, cells recorded during the night rarely showed evidence of PPD. PPD is a widely observed coding mechanism in neural circuits and has been observed in synapses between SCN and paraventricular neurons (Wang et al., 2003). Given the strong diurnal rhythm of PPD, GABAergic communication between SCN neurons is more complex than previously thought.

Short-term synaptic depression is the activity-dependent reduction in synaptic transmission that occurs within several hundred milliseconds of the onset of activity (Zucker, 1989). This type of synaptic plasticity acts as a temporal filter of presynaptic action potential firing frequency in that neurotransmission at particular frequency ranges is depressed while other frequencies transmit information reliably (Fortune and Rose, 2001). The paired-pulse depression presented in this study indicates that intra-SCN synapses generally allow frequencies at or above the average daytime firing frequency to pass information during the late day and night, yet these frequencies are rejected during the early day. This allows those frequencies to be subject to varying degrees of gain control over the course of the day which may affect the code of the synaptic signal received by the postsynaptic cell (Abbott et al., 1997). Recent evidence suggests that intra-SCN synaptic neurotransmission can change postsynaptic firing from a regular to an irregular pattern (Kononenko and Dudek, 2004). In light of this, the rate of presynaptic spiking could be considered an important determinant of the neural coding

that controls postsynaptic firing patterns in the SCN, and synaptic plasticity could reasonably be expected to modulate this signaling.

Presynaptic inhibition

PPD may result from either presynaptic release modulation or postsynaptic $GABA_A$ receptor desensitization. A diurnal rhythm in the $GABA_A$ receptor subunit composition in SCN neurons is suggested by a diurnal variation in the strength of Zn^{2+} inhibition of the $GABA_A$ receptor (Kretschmannova et al., 2003). While such a postsynaptic mechanism could contribute to changes in synaptic strength, our data indicate that the diurnal regulation of short-term synaptic plasticity occurs presynaptically. Type 1 PPD was shown to be a release-dependent phenomenon (Fig. 3A) and paired focal application of muscimol as well as an analysis of GABA release probability (see results) indicates that Type 2 PPD also has a presynaptic site of action. As described in previous studies (Jiang, Yang, and Allen, 1997b), the majority of currents recorded here (see methods) are expected to have been evoked from neighboring SCN cells, implying that presynaptic SCN cells control the expression of PPD. Thus, the transmission of presynaptic action potential firing frequency is at least partly filtered at the level of neurotransmitter release.

Vesicle depletion

Two of the most common mechanisms producing PPD in other systems are depletion of the readily releasable pool of synaptic vesicles and negative feedback mediated by presynaptic $GABA_B$ receptors (Zucker and Regehr, 2002). The majority of Type 1 PPD recorded during the early day exhibited paired currents with relative

amplitudes consistent with a depletion of the readily releasable pool of neurotransmitter (Fig. 3A). Interestingly, glutamic acid decarboxylase (GAD) content (Cagampang et al., 1996) and activity as well as GABA content in the SCN are higher during the night when Type 1 PPD is not observed (Aguilar-Roblero et al., 1993). Several questions need to be answered to determine if this rhythm of GABA content contributes to PPD. First, it will be important to know if total GABA content in the SCN translates into lower GABA content in vesicles at the axon terminal. Second it would be necessary to determine if the relatively lower amounts of GABA during the early daytime hours contribute to fewer readily-releasable vesicles (Schneggenburger et al., 2002), or a lowered rate of vesicle recycling (Stevens and Wesseling, 1998). Finally, it is interesting to note that the GABA rhythm is blocked in the absence of retinal light inputs (Huhman et al., 1999). Therefore, it might be possible to test the hypothesis that type 1 PPD depends upon SCN GABA levels in animals held in constant darkness.

Presynaptic $GABA_B$ autoreceptors

Another common mechanism of PPD is presynaptic inhibition via G-protein coupled receptors (i.e. $GABA_B$ receptors). This mechanism has been described at the SCN-Paraventricular nucleus (PVN) synapse (Cui et al., 2000). In both of these earlier studies, the inter-stimulus intervals (150 msec) used and the maximal PPD ratio observed (0.66 ± 0.05) were similar to the results reported here. Although presynaptic $GABA_B$ autoreceptor activation has been shown to inhibit GABA release presynaptically in SCN cell cultures (Chen and van den Pol, 1998), our results do not support a role of presynaptic $GABA_B$ receptors in intra-SCN paired-pulse depression or in the modulation of GABA release.

Hence, our results suggest that the SCN-PVN synapse may be regulated in fundamentally different ways from intra-SCN synapses. PVN-projecting axons may arise from neurons that do not participate in SCN-SCN communication (Van den Pol, 1980), or axon branches terminating within the SCN may not express $GABA_B$ receptors while axon branches terminating at the PVN do. Experiments performed in the presence of NEM also support a lack $GABA_B$ receptor involvement. To determine whether other G-protein coupled receptors—i.e. any transmitter co-released with GABA—might be involved in PPD in the SCN will require additional experiments.

Possible mechanisms

Although PPD found at the faster range of endogenous daytime action potential firing frequencies (25 msec inter-stimulus interval, 50 Hz) appears to depend on use-dependent vesicle depletion, the mechanism responsible for PPD near the mean frequency of endogenous daytime action potentials (200 msec inter-stimulus interval, 5 Hz) remains unclear. Reducing external $[Ca^{2+}]$ leads to a reduced Ca^{2+} influx at axon terminals, which consequently reduces neurotransmitter release probability. Here, reducing external $[Ca^{2+}]$ also reduced PPD at the 200 msec interval, implying a Ca^{2+}-dependent mechanism. Recent evidence suggests that SNAP 25 and Munc 18, proteins known to be involved in synaptic vesicle docking (Thomson, 2000) have expression cycles with a peak around CT 10 (Voets et al., 2001;Panda et al., 2002a). This suggests that lower levels of these proteins might exist earlier in the day, and could contribute to the reduced neurotransmitter release probability.

In summary, our data show that synaptic plasticity exhibits a diurnal rhythm in the local SCN circuitry, implying that neurotransmission within this local circuitry is

under the control of the circadian clock. The frequencies at which this can be observed correspond to the spontaneous daytime action potential firing frequencies observed in SCN neurons during the daytime. Two separate mechanisms may be responsible for the expression of this circadian rhythmicity in synaptic plasticity. Therefore, both frequency and synaptic strength of intra-SCN GABAergic neurotransmission are likely to signal a cell's relative circadian phase to neighboring neurons. This is likely to contribute to the overall synchronization of endogenous circadian rhythms within the SCN.

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Manuscript 2

Retrograde suppression of the GABAergic current in a subset of suprachiasmatic nucleus (SCN) neurons

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Abstract

Retrograde signaling is a process by which a postsynaptic neuron releases a transmitter that modulates presynaptic neurotransmitter release. In the SCN, the release of a neurotransmitter from the dendrites is suggested by the presence of dendritic dense core vesicles. Spontaneous and evoked GABAergic currents were inhibited in a subpopulation of SCN cells (40% daytime, 29% nighttime) following a train of depolarizations with a frequency similar to that of the endogenous daytime action potential firing frequency. We further show that the retrograde messenger mediating this inhibition is released following an increase of the postsynaptic Ca^{2+} partially mediated by L-type Ca^{2+} channels, and acts on a presynaptic pertussis-toxin sensitive G-protein coupled receptor. Since a majority of SCN neurons receive GABAergic input from neighboring cells, these results describe a mechanism by which a subset of SCN neurons can inhibit GABAergic synaptic input.

Introduction

Retrograde neurotransmission has been described as a precise, rapid mechanism by which postsynaptic cells can regulate the activity of specific presynaptic inputs (Alger, 2002; Kreitzer and Regehr, 2001; Pitler and Alger, 1992). Candidates for retrograde transmitters include dopamine, endocannabinoids, and neuropeptides such as oxytocin and vasopressin. In hippocampal neurons, action potentials depolarize the neural membrane and activate a Ca^{2+} influx through voltage-gated Ca^{2+} channels that triggers the release of endocannabinoids (Lenz and Alger, 1999; Pitler and Alger, 1992; Pitler and Alger, 1994). Similarly, cerebellar Purkinje cells release endocannabinoids that inhibit excitatory synaptic inputs from the climbing and parallel fibers (Kreitzer and

Regehr, 2001). The endocannabinoids are synthesized when needed and are immediately released.

In the hypothalamus, retrograde transmitters are released from membrane bound vesicles. For example, magnocellular neurons in the supraoptic and paraventricular nuclei release the peptides vasopressin and oxytocin from dense-core vesicles located in somato-dendritic compartments (Ludwig and Pittman, 2003). In the hypothalamic suprachiasmatic nucleus (SCN), dendritic dense-core vesicles are located in the dendrites of a subpopulation of neurons. Following membrane depolarization, the exocytosis of these dense-core vesicles could be observed at nonsynaptic sections of the dendrites (Castel et al., 1996). These observations led Castel et al. (1996) to propose that neuromodulatory substances could be released from the dendrites of SCN neurons.

Intra-SCN synapses are almost entirely GABAergic with a majority of SCN neurons expressing the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) and $GABA_A$ receptors expressed on both postsynaptic specializations and presynaptic axon terminals (Belenky et al., 2003; Jiang et al., 1997; Kim and Dudek, 1992; Moore and Speh, 1993). GABA mediated neurotransmission contributes to synchronization of SCN action potential firing (Liu and Reppert, 2000; Shirakawa et al., 2000). GABA may act as an excitatory neurotransmitter during some portions of the circadian cycle and an inhibitory neurotransmitter during other portions of the circadian cycle, a finding that has been highly controversial (De Jeu and Pennartz, 2002; Gribkoff et al., 2003; Gribkoff et al., 1999; Wagner et al., 1997). Recently, GABA has been shown to control the regularity of action potential firing (Kononenko and Dudek, 2004). Since many SCN neurons fire action potentials faster during the day than during the night (Green and Gillette, 1982; Inouye and Kawamura, 1979), retrograde signaling

could provide SCN neurons the ability to regulate synaptic inputs in a circadian manner. We therefore performed experiments to test the hypothesis that SCN neurons can regulate GABAergic synaptic inputs via a retrograde signal.

Here we demonstrate that a subset of SCN neurons inhibit the frequency and amplitude of $GABA_A$-mediated currents following postsynaptic depolarization. This inhibition depends upon elevated levels of postsynaptic Ca^{2+} that are partially mediated by the L-type voltage-activated Ca^{2+} channel, similar to the retrograde signaling observed in the hippocampus and cerebellum (Brenowitz and Regehr, 2003; Lenz and Alger, 1999; Pitler and Alger, 1992). We also show that the inhibition is mediated by a presynaptic pertussis toxin sensitive G-protein coupled receptor, which may provide clues about the identity of the retrograde messenger used in the SCN.

Materials and Methods

Preparation of brain slices

Male Sprague-Dawley rats (6-8 wk old) were maintained on a 12 hr light/12 hr dark lighting schedule for at least one week. The Institutional Animal Care and Use Committee of OHSU approved all experimental procedures involving animals. Coronal (250 μm thick) hypothalamic slices containing the SCN were prepared on a vibrating blade microtome (Leica VT1000S, Nussloch, Germany) and transferred to the recording chamber where they were submerged in 32°C ACSF as described in detail previously (Gompf and Allen, 2004). Experiments were performed with the SCN visualized using infrared differential interference microscopy (IR-DIC). A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) attached to a stimulus isolation unit (Grass Instruments, Quincy, MA) was placed into the lateral SCN, approximately 100 – 200

μm dorsal and lateral to the recording site in the ventromedial SCN. The stimulus amplitude (20 – 50 V) was set to the minimum value that reliably evoked a current, and currents were evoked at no more than 1 Hz to avoid paired-pulse depression (Gompf and Allen, 2004).

Patch clamp recording

Whole-cell patch clamp recordings were performed 0.5 to 10 hr after slice preparation in neurons located in the ventromedial portion of the SCN as described in detail elsewhere (Gompf and Allen, 2004). The microelectrode solution contained (in mM): $CsCH_3O_3S$ 100, CsCl 60, HEPES 10, $MgCl_2$ 1, ATP 3, 2(triethylamino-N-(2,6-dimethyl-phenyl)acetamide (QX-314) 5, pH 7.2 (Lenz and Alger, 1999). The holding potential for whole cell voltage clamp recordings was -80 mV except during the depolarization protocol in which the cell was depolarized to +20 mV either once for 10 sec or ten 100 msec duration pulses at 5 Hz. No correction was made for the liquid junction potential. GABA-mediated currents were pharmacologically isolated by bath application of the selective AMPA receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 μM) and the selective NMDA receptor blocker DL-2-Amino-5-phosphonopentanoic acid (APV, 50 μM). The currents were identified as being mediated by $GABA_A$ receptors since they could be blocked by picrotoxin (50 μM). Some experiments were performed following a 20-minute preincubation with N-ethylmaleimide (NEM, 200 μM), the selective L-type Ca^{2+} channel antagonist nimodipine (5 μM), or the selective L-type Ca^{2+} channel agonist Bay K8644 (5 μM). All drugs were purchased from Sigma (St. Louis, MO).

Results

Inhibition of GABAergic current frequency and amplitude by postsynaptic depolarization

Spontaneous $GABA_A$-mediated currents, recorded during the daytime in the presence of APV (50 μM) and CNQX (5 μM), had a frequency of 5.0 ± 0.9 Hz (range: 2 – 15 Hz, n = 14, Fig. 6A before arrow). In 7 of these cells (50%), spontaneous current frequency dropped to 2.9 ± 0.9 Hz ($p = 0.028$, Wilcoxon signed rank test) during the first two seconds following depolarization of the postsynaptic cell (10 sec, – 80 mV to +20 mV, Fig. 6B). Average current amplitude during the two second period following depolarization was also reduced compared to the two second period prior to depolarization (26.7 ± 1.5 pA, average of 4-31 currents, vs. 19.8 ± 0.8 pA, average of 2-14 currents; n = 7 cells, $p = 0.08$, paired t-test). The GABAergic current frequency returned to control values (4.5 ± 0.6 Hz) 20 sec after the membrane depolarization.

To examine this suppression of synaptic transmission in more detail, $GABA_A$-mediated currents were evoked using a concentric bipolar stimulating electrode placed into the SCN (50 μM APV, 5 μM CNQX). Currents were evoked at 0.5 Hz, a frequency that does not elicit short-term synaptic plasticity in GABAergic synapses in the SCN (Gompf and Allen, 2004). Simulating short trains of action potentials, postsynaptic cells were depolarized (-80 mV to +20 mV) ten times for 100 msec at 5 Hz. Under these conditions, the amplitude of the evoked GABAergic currents was significantly reduced in 25 of 63 cells (40%) during daytime recordings (ZT 2 – 10, Fig. 7A), and 4 of 14 (29%) of cells during nighttime recordings (ZT 14 - 19). These results indicate that postsynaptic depolarization leads to a reduction of the $GABA_A$ receptor-mediated current amplitude in a subset of SCN neurons regardless of the time

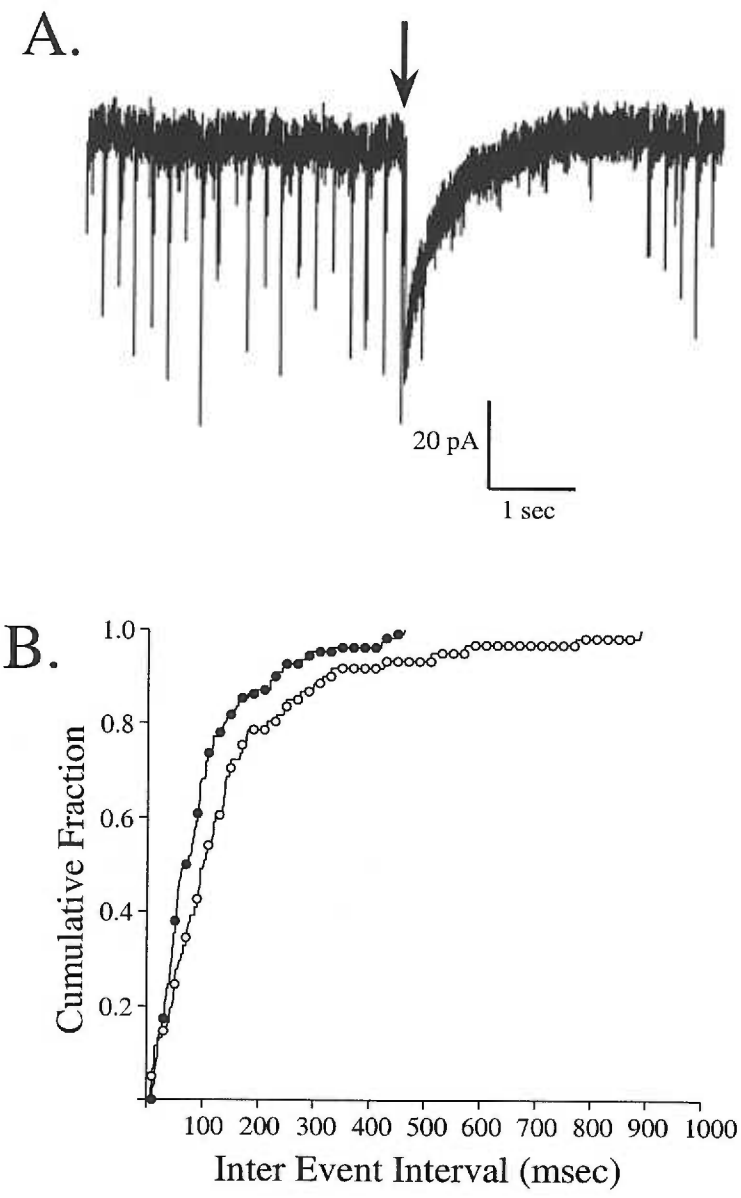

Figure 6: Inhibition of spontaneous IPSCs following postsynaptic depolarization. A. Example of spontaneous inhibitory postsynaptic currents (sIPSC), recorded from a neuron in the ventromedial SCN in the presence of CNQX (5 μM) and D-APV (50 μM). sIPSCs were recorded before and after a step depolarization (10 sec from –80 mV to +20 mV) indicated by the arrow. Inhibition of both amplitude and frequency of sIPSCs begins almost immediately following depolarization and lasts for a number of seconds. B. Cumulative fraction histogram of 7 cells with reduced sIPSC frequency following depolarization. Closed circles denote sIPSC frequency prior to depolarization and open circles sIPSC frequency following depolarization. The longer inter-event intervals in the open circle data indicate a reduction in sIPSC frequency following postsynaptic depolarization.

of recording. However, the SCN cells recorded display a slight preference for exhibiting depolarization-induced inhibition during the day. Due to the possibility of excitatory GABAergic neurotransmission in the SCN (Wagner et al., 1997) this reduction of the GABAergic current was not called by the commonly used term "depolarization-induced suppression of the inhibitory current (DSI)" but rather "depolarization-induced inhibition of the GABAergic current" (De Jeu and Pennartz, 2002).

Duration of depolarization-induced inhibition at SCN synapses

To determine the time course of recovery from the depolarization-induced inhibition, currents were evoked as described above at 0.5 Hz. Average amplitudes for the 20 sec prior to 10 postsynaptic depolarizing pulses (100 msec, 5 Hz) were compared to the average amplitudes during the 30 sec. following the depolarizations (Fig. 7B). The time constant of recovery from inhibition was similar in cells recorded during the day ($\tau = 3.2$ sec, $R = 0.94$) and the night ($\tau = 3.6$ sec, $R = 0.84$). Also, the amplitudes of the first currents (2 sec. interval) following depolarization were similar in cells recorded during the day (43.3 ± 6.1 % of control) and the night (45.1 ± 4.1 % of control), indicating that the strength of depolarization-mediated inhibition is constant over different recording times. In the following experiments, we compared the effects of different treatments by measuring the magnitude of inhibition during the first 12 seconds (6 evoked currents) following postsynaptic depolarization on cells recorded during the day.

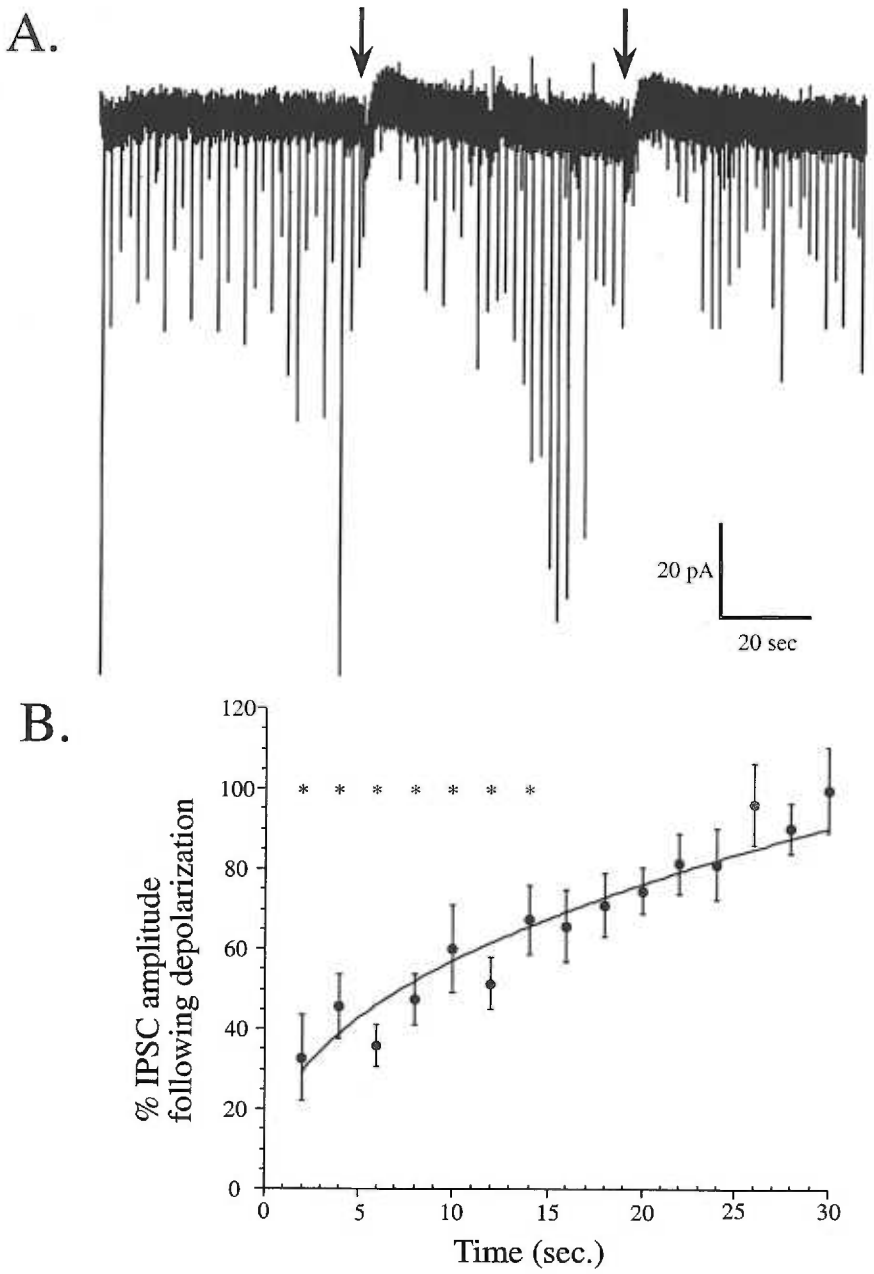

Figure 7: The timecourse of inhibition of evoked IPSCs: A. Example trace of IPSCs evoked using a concentric bipolar stimulating electrode placed approximately 100 μm dorsal and lateral to the site of recording. IPSCs were evoked at a frequency of 0.5 Hz, arrows show the times at which the postsynaptic cell was depolarized (10 pulses from –80 mV to +20 mV, 100 msec duration, 5 Hz). Note the repeatable reduction in IPSC amplitude lasting for approximately 20 seconds following depolarization. B. The average of three current amplitudes at each time point following depolarization (2-30 sec every other second) for each of 10 cells is expressed as % amplitude relative to the average current amplitude of all IPSCs 20 sec. prior to depolarization (5 Hz, see Fig. 2A). Values are expressed in mean ± SE, maximum inhibition = 67.3 ± 10.7 % at the IPSC following two seconds after the depolarization, _ of return to average pre-depolarization values = 4 sec. Asterisks denote a significant ($p < 0.05$) difference from cells not expressing DSI at those points following depolarization. On the basis of these data, it was decided to compare treatments by comparing the average inhibition of the first 5 IPSCs (first 10 sec) following depolarization.

Postsynaptic depolarization produces an inhibition of presynaptic GABA release

Experiments were designed to determine whether the postsynaptic depolarization could alter the activity of $GABA_A$ receptors leading to the observed inhibition of evoked GABAergic currents. To explore this question, the amplitudes of muscimol (100 μM, a $GABA_A$ agonist) induced currents were measured before and after a 10 second depolarization (voltage step to +20 mV from a holding potential of –80 mV). In the nine cells examined (6 that showed depolarization-induced inhibition of the spontaneous GABAergic currents, three that did not), no difference in the muscimol-induced current amplitude was found (118 ± 31 pA before vs. 109 ± 24 pA following depolarization $p = 0.3$, paired t-test). These data confirm that the inhibition of GABAergic currents induced by membrane depolarization was not due to an alteration in the activity of postsynaptic $GABA_A$ receptors.

Additionally, we examined whether the depolarization-induced inhibition reduced the presynaptically mediated paired-pulse depression observed at intra-SCN synapses (Gompf and Allen, 2004). Paired-pulse depression was measured at 200 msec inter-stimulus intervals in the presence of APV (50 μM) and CNQX (5 μM), and defined as a paired-pulse ratio (current2 / current1) significantly less than 1 (Gompf and Allen, 2004). The average of five pairs of evoked currents from cells that exhibited both paired-pulse inhibition and depolarization-induced inhibition (1 second intervals between current pairs, $n = 9$) was measured before and after depolarization of the recorded neuron (10 sec voltage step to +20 mV from a holding potential of –80 mV). The average paired-pulse ratio in these cells was 0.77 ± 0.03 before depolarization and 1.1 ± 0.1 following depolarization (Fig. 8A). These data are consistent with the

postsynaptic depolarization inducing a reduction of GABA release via a presynaptic mechanism.

Presynaptic G-protein coupled receptor involvement

Since presynaptic inhibition is often mediated by G-protein coupled receptors, we investigated a role for pertussis-toxin sensitive G-protein coupled receptors. Five cells exhibiting depolarization-induced inhibition (current amplitudes 42.3 ± 4.0% of control) were exposed to the pertussis-toxin sensitive G-protein uncoupling agent N-ethylmaleamide (NEM, 200 μM) for 20 min and then retested (Fig. 8B). NEM blocked the depolarization-induced inhibition of the evoked GABAergic currents. Current amplitudes following depolarization were 5.0 ± 3.5 % greater than control in the presence of NEM, significantly different from the depolarization-induced inhibition recorded from these cells prior to NEM exposure ($p = 0.000001$, paired t-test). Rundown of the GABA current during the long NEM exposure period was not a factor in these experiments since control GABAergic current amplitudes prior to depolarization were not different (41.6 ± 4.5 pA) from those recorded during NEM application (40.9 ± 6.5 pA, $p = 0.89$, paired t-test). Thus, the retrograde messenger likely acts on a presynaptic pertussis toxin-sensitive G-protein coupled receptor.

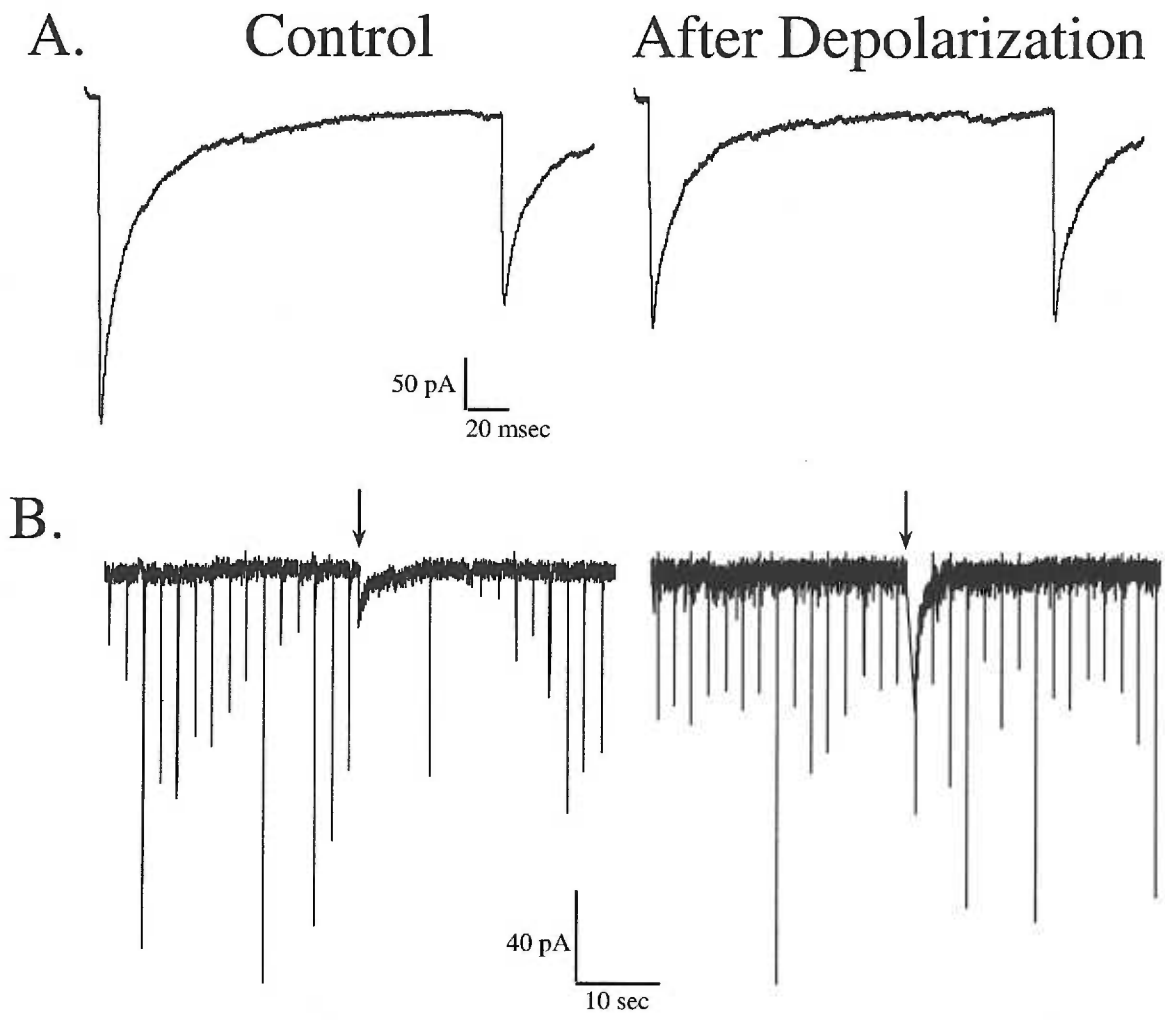

Figure 8: Inhibition is mediated at a presynaptic G-protein coupled receptor. A. Average of 5 traces of a cell exhibiting both paired-pulse depression and DSI during the early subjective day (ZT 3.5). Following depolarization, IPSC1 amplitudes decreased, thereby abolishing the presynaptically mediated paired-pulse depression. B. IPSCs evoked and depolarization performed in the same fashion as in Fig. 2A, in the presence (right) and absence (left) of the pertussis-toxin sensitive G-protein uncoupling agent NEM (200 μM). The lack of effect of depolarization on IPSC amplitude indicates a presynaptic G-protein coupled receptor mechanism.

Postsynaptic Ca^{2+} influx mediates depolarization-induced inhibition

Retrograde signaling in the hippocampus and cerebellum is dependent on the entry of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels (Brenowitz and Regehr, 2003; Lenz and Alger, 1999; Lenz et al., 1998). In each of the experiments described thus far in this work, the internal solution used contained no Ca^{2+} buffer. To test the Ca^{2+} dependence of depolarization-induced inhibition in the SCN, evoked current amplitudes were measured in 6 cells using the Ca^{2+} buffer ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 11 mM) in the internal solution. Of these six cells, at least two cells (40% daytime recordings, see above) would have been expected to show depolarization-induced inhibition; however, the GABAergic current amplitudes did not change after depolarization (5.4 ± 5.4 % of control current amplitude) in any of the cells examined. Therefore, Ca^{2+} influx through voltage-activated channels may be required for retrograde signaling in the SCN.

In the SCN, L-type Ca^{2+} channels are involved in action potential firing (Cloues and Sather, 2003; Pennartz et al., 2002), and we hypothesized that these channels could contribute to depolarization-induced inhibition. To test this hypothesis, we exposed 7 cells exhibiting retrograde inhibition to the L-type Ca^{2+} channel antagonist nimodipine (5 μM). Nimodipine partially blocked the depolarization-induced inhibition of the GABAergic current. Current amplitudes following depolarization in the presence of nimodipine were suppressed relative to control (87.1 ± 0.8 % of control amplitude vs 50.8 ± 3.1 % in the absence of nimodipine, Fig. 9A, C), which is significantly less depolarization-induced inhibition than in the absence of nimodipine ($p = 0.0009$, paired t-test). To further evaluate the role of L-type Ca^{2+} channels, we exposed cells expressing depolarization-induced inhibition to the L-type voltage-activated Ca^{2+}

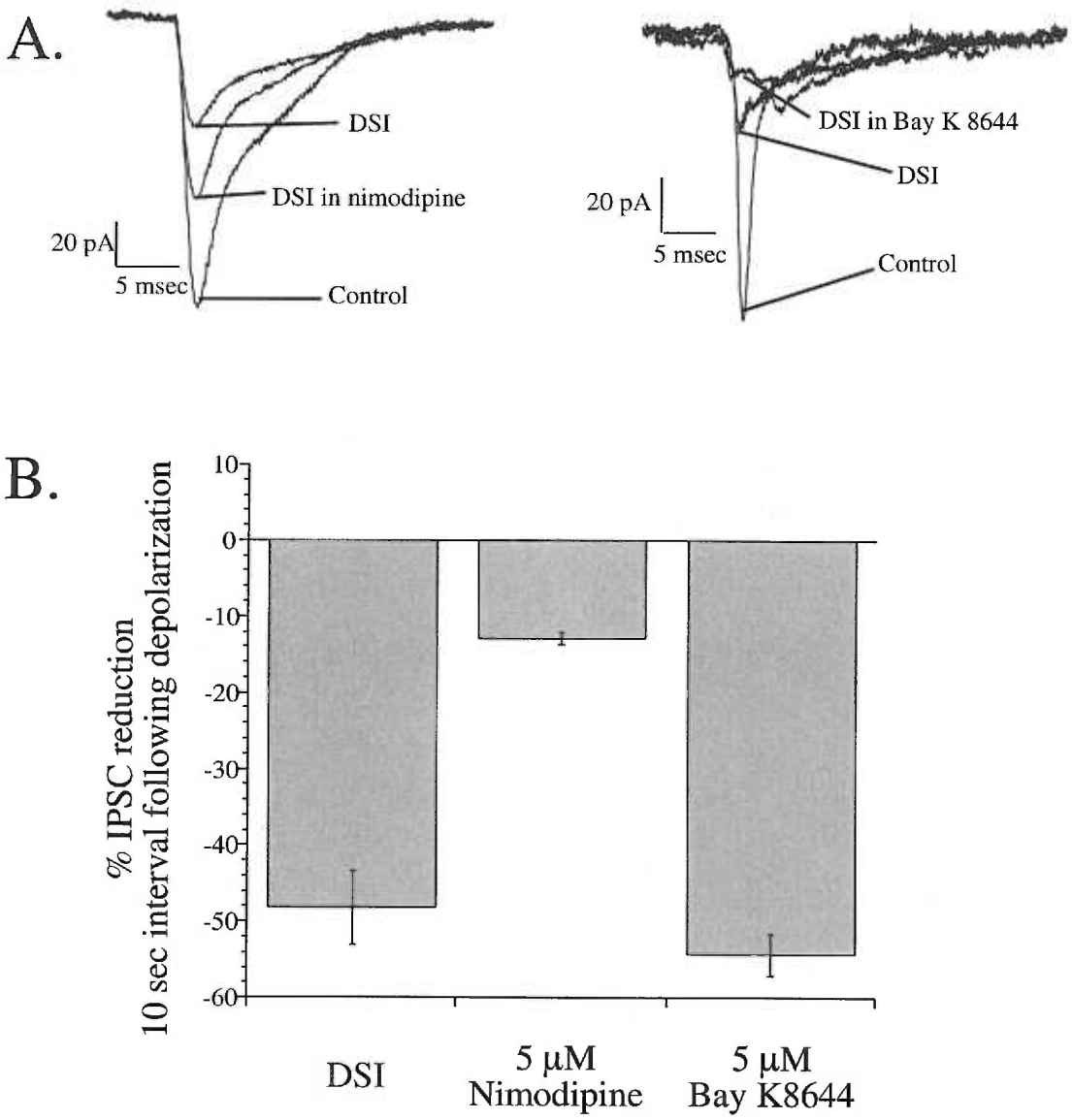

Figure 9: Postsynaptic Ca^{2+} influx through L-type Ca^{2+} channels partially mediates retrograde messenger release. A. Average current amplitudes of the first 6 currents recorded before (control) or after depolarization (DSI). The L-type Ca^{2+} channel antagonist nimodipine (5 μM) partially inhibits DSI (left), while the L-type Ca^{2+} channel agonist Bay K8644 (5 μM) enhances DSI (right). B. Reversal of DSI by nimodipine and enhancement by Bay K8644. The average reduction in IPSC amplitude following depolarization (49.2 ± 3.1 %, see 3 A. above) from twelve cells was partially reversed in 7 cells by 5μM Nimodipine (13 ± 0.8 %, n = 7). The average reduction in IPSC amplitude was enhanced in the presence of 5 μM Bay K 8644 to 54.3 ± 2.8 % (n = 5). Values represent mean ± SE.

channel specific agonist Bay K8644 (5 μM). In 5 out of 6 cells, the strength of depolarization-induced inhibition was enhanced slightly so that post-depolarization current amplitudes in the presence of Bay K8644 were 45.7 ± 2.8 % of control vs. 51.8 ± 3.1 % of control in the same cells prior to Bay K8644 exposure ($p = 0.04$, Fig. 9B, C). These data provide evidence for a retrograde signaling pathway involving postsynaptic L-type Ca^{2+} channels.

Discussion

A subpopulation of SCN neurons uses a retrograde signaling pathway to regulate GABAergic synaptic inputs. This inhibition occurs as a result of postsynaptic Ca^{2+} influx following postsynaptic membrane depolarization and involves the release of a retrograde messenger that acts on a presynaptic pertussis-toxin-sensitive G-protein coupled receptor. Retrograde signaling in the SCN demonstrates a modulatory mechanism similar to the retrograde neurotransmission observed in the hippocampus and cerebellum (Alger, 2002; Kreitzer and Regehr, 2001). Onset of inhibition of GABAergic current amplitude and frequency was rapid, occurring within a second or less following depolarization, and lasting for up to 20-30 seconds (Fig. 1). One aspect that might distinguish retrograde inhibition in the SCN, however, is the possibility that it is mediated by a transmitter contained in the previously described dendritic dense-core vesicles (Castel et al., 1996). The identity of the retrograde transmitter is an important question for future study. Endocannabinoids have been identified as a retrograde neurotransmitter in the hippocampus and cerebellum (Alger, 2002). However, cannabinoid receptor binding is particularly sparse in the SCN (Herkenham et al., 1991), and dendritic peptidergic vesicles mediating retrograde inhibition are somewhat

more common in the hypothalamus (Ludwig and Pittman, 2003). The dendritic vesicles may contain any of the peptides known to be expressed in the SCN - vasoactive intestinal protein (VIP), arginine-vasopressin, somatostatin, or gastrin releasing peptide (GRP) - thus providing a novel role for one or several of these peptides not only in circadian physiology but also in expanding the number of modulators involved in retrograde signaling (Van den Pol and Gorcs, 1986; Van den Pol and Tsujimoto, 1985).

One noteworthy aspect of depolarization-induced inhibition is that the percentage of cells exhibiting depolarization-induced inhibition was slightly higher when a single step depolarization of longer duration was applied (50 % of cells during spontaneous current recordings) than when multiple depolarizations were used at a frequency of 5 Hz (40 % of cells during the timecourse of inhibition recordings). This suggests that a threshold number of action potentials may be required to elicit depolarization-induced inhibition and that a threshold Ca^{2+} concentration is required to bring about retrograde messenger release. Consequently, the release of retrograde transmitter may be sensitive to Ca^{2+} levels from a number of sources, including those under the control of the circadian clock (Ikeda et al., 2003). The L-type Ca^{2+} channel current is also subject to diurnal modulation in the SCN, peaking during the day, coincident with the elevated action potential firing frequency (Pennartz et al., 2002). In this study we have found that Ca^{2+} entering through the L-type channel partially mediates retrograde inhibition. A possible reason why only a minor diurnal variation was found here is that whole cell patch clamp recordings might dilute the priming effect of elevated baseline Ca^{2+} levels or increased L-type channel activity. Also, a number of other voltage-activated Ca^{2+} channels that are not diurnally modulated might be involved in elevating postsynaptic Ca^{2+} levels sufficiently to induce retrograde inhibition in the

SCN, a hypothesis, which would be difficult to examine due to the possible role of these channels in GABA release.

In conclusion, we have found that retrograde signaling by a subpopulation of SCN neurons modulates GABAergic synaptic neurotransmission. This suppression of the $GABA_A$ receptor-mediated current may play a role in shaping action potential firing patterns and due to its partial mediation by the L-type Ca^{2+} channel, may be an important daytime synaptic modulatory mechanism.

Acknowledgments

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Manuscript 3

Nociceptin/orphanin FQ (N/OFQ) inhibits excitatory and inhibitory synaptic signaling in the suprachiasmatic nucleus (SCN)

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Abstract

Environmental synchronization of the endogenous mammalian circadian rhythm involves glutamatergic and GABAergic neurotransmission within the hypothalamic suprachiasmatic nucleus (SCN). The neuropeptide nociceptin/orphanin FQ (N/OFQ) inhibits light-induced phase shifts, evokes K^+-currents and reduces the intracellular Ca^{2+} concentration in SCN neurons. Since these effects are consistent with a modulatory role for N/OFQ on synaptic transmission in the SCN, we examined the effects of N/OFQ on evoked and spontaneous excitatory and inhibitory currents in the SCN. N/OFQ produced a consistent concentration-dependent inhibition of glutamate-mediated excitatory postsynaptic currents (EPSC) evoked by optic nerve stimulation. N/OFQ did not alter the amplitude of currents induced by application of AMPA or NMDA nor the amplitude of miniature excitatory postsynaptic currents (mEPSC) consistent with a lack of N/OFQ effect on postsynaptic AMPA or NMDA receptors. N/OFQ significantly reduced the mEPSC frequency. The inhibitory actions of N/OFQ were blocked by ω-conotoxin GVIA, an N-type Ca^{2+}channel antagonist and partially blocked by ω-agatoxin TK, a P/Q type Ca^{2+} channel blocker. These data indicate that N/OFQ reduces evoked EPSC, in part, by inhibiting the activity of N- and P/Q-type Ca^{2+} channels. In addition, N/OFQ produced a consistent reduction in baseline Ca^{2+} levels in presynaptic retinohypothalamic tract (RHT) terminals. N/OFQ also inhibited evoked $GABA_A$ receptor mediated inhibitory postsynaptic currents (IPSC) in a concentration dependent manner. However, N/OFQ had no effect on currents activated by muscimol application or on the amplitude of miniature IPSC (mIPSC) and significantly reduced the mIPSC frequency consistent with an inhibition of GABA release downstream from Ca^{2+} entry. Finally, N/OFQ inhibited the paired-pulse

depression observed in SCN GABAergic synapses consistent with a presynaptic mechanism of action. Together these results suggest a widespread modulatory role for N/OFQ on the synaptic transmission in the SCN.

Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the site of an endogenous pacemaker that controls mammalian circadian rhythms. The biological clock does not keep perfect 24-hour time; nor are the length of the natural light and dark periods constant through the seasons. Evolution has favored those organisms that have their circadian clock entrained to the environmental light-dark cycle (DeCoursey et al., 1997, Ouyang et al., 1998). The retina is essential to generate phase shifts that entrain the circadian clock to environmental cycles. Light can shift the circadian clock producing phase delays early in the night, phase advances late in the night and no phase change during the day. The anatomical pathway that communicates environmental light information to the circadian clock is the retinohypothalamic tract (RHT), a direct projection from a subset of retinal ganglion cells to the SCN (Moore and Lenn, 1972, Berson et al., 2002). Neurochemically it is glutamate released from RHT terminals acting on both AMPA and NMDA receptors that entrains the circadian clock (Colwell and Menaker, 1992, Ding et al., 1997, Mintz et al., 1999). Afferent pathways from other brain regions have been described that also modulate the light signal to the SCN. For example, it has been observed that 5-HT can inhibit light-induced phase shifts by activating presynaptic receptors located on RHT terminals (Pickard et al., 1996, Pickard and Rea, 1997, Pickard et al., 1999). Similarly, activation of $GABA_B$ receptors located

on RHT terminals will inhibit light-induced phase shifts (Ralph and Menaker, 1989). Activation of either 5-HT or $GABA_B$ receptors reduces the release of neurotransmitter from the presynaptic axon terminals (Jiang et al., 1995, Pickard et al., 1999, Jiang et al., 2000).

The neuropeptide nociceptin/orphanin FQ (N/OFQ), when microinjected into the SCN, also inhibits the phase advance induced by a light pulse in the late night (Allen et al., 1999). N/OFQ and its receptor (ORL1 receptor) are involved in numerous biological functions including pain transmission (Mogil et al., 1996, Heinricher et al., 1997), stress (Jenck et al., 1997, Meis and Pape, 2001), learning and memory (Amano et al., 2000), and locomotor activity (Devine et al., 1996). One commonly observed mechanism of action of N/OFQ in these systems is a reduction of neurotransmitter release via inhibition of voltage-activated Ca^{2+} channels (Knoflach et al., 1996). We therefore hypothesized that the glutamate-mediated synaptic transmission from the retina would be reduced by N/OFQ receptors located on RHT terminals.

GABAergic synapses between SCN neurons are also involved in phase shifting the circadian clock (Smith et al., 1989, Golombek and Ralph, 1994, Tominaga et al., 1994) and bicuculline, a $GABA_A$ antagonist, blocks the light-induced phase delays (Ralph and Menaker, 1989, Gillespie et al., 1997). These data suggest that part of N/OFQ's effect on the light-induced phase shift may involve modulation of GABAergic synaptic transmission. Consequently, we postulated that the release of GABA, the principle neurotransmitter at intra-SCN synapses (Kim and Dudek, 1992, Moore and Speh, 1993, Jiang et al., 1997), might be inhibited by N/OFQ.

By recording evoked and spontaneous synaptic currents in acute brain slice preparations, we show that N/OFQ suppresses glutamate- and GABA-release in a dose-

dependent manner during daytime and nighttime recordings. Presynaptic RHT inhibition was shown to involve both a reduction in baseline Ca^{2+} levels and a direct inhibition of voltage-activated N-type and P/Q-type Ca^{2+} channels. These results demonstrate that N/OFQ is an important neuropeptide regulating synaptic neurotransmission in the SCN.

Materials and Methods:

Preparation of brain slices

Male Sprague-Dawley rats (6-8 wk old) were maintained on a 12 hr light/12 hr dark schedule for at least 2 weeks before recording. During the light phase, rats were deeply anesthetized with halothane, their brains removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 20, $MgCl_2$ 4, CaC l_2 0.5, and glucose 11 saturated with 95% O_2/5% CO_2. Coronal (250 - 300 μm thick) hypothalamic slices containing the SCN were prepared on a vibratome (Leica VT 1000S, Nussloch, Germany), and transferred to the recording chamber in which they were completely submerged in 32°C ACSF (same as above, except $MgCl_2$ 1.2, $CaCl_2$ 2.4 mM) or 25°C ACSF for presynaptic Ca^{2+} fluorescence experiments (see below). The Institutional Animal Care and Use Committee of OHSU approved all experimental procedures involving animals and all efforts were made to minimize pain and the numbers of animals used.

Patch clamp recording

Experiments were performed with the SCN visualized using infrared differential interference microscopy (IR-DIC). A concentric bipolar stimulating electrode (FHC,

Bowdoinham, ME) attached to a stimulus isolation unit (Grass Instruments, Quincy, MA) was placed either directly into the SCN (IPSC recordings) or into the optic chiasm (EPSC recordings). Whole-cell patch clamp recordings were performed 0.5 to 10 hr after slice preparation. Whole-cell patch electrodes had resistances of 5 - 10 MΩ when filled with a solution containing (in mM): $CH_3O_3SC_5$ 87, CsCl 20, $CaCl_2$ 1, HEPES 10, EGTA 11, CsOH 25, ATP 3, GTP 0.3, QX-314 0.5, pH 7.4. The electrode was advanced into the brain slice and a seal with the cell membrane (resistance: 2-15 GΩ) was obtained by applying negative pressure. The cell membrane was ruptured, currents were measured with an Axopatch-1D amplifier (Axon Instruments, Union City, CA) and sampled at 10 kHz using the data acquisition program PulseFit (HEKA, Lambrecht, Germany) on a Macintosh computer. The holding potential for whole cell voltage clamp recordings was –60 mV, no correction was made for the liquid junction potential.

Synaptic currents were evoked by electrical stimulation (0.2 msec; 10 - 60 V). Glutamate-mediated currents were pharmacologically isolated by bath application of picrotoxin (50 μM) and their identity confirmed by blocking with CNQX (5 μM) and APV (50 μM) in selected experiments. $GABA_A$-mediated currents were pharmacologically isolated by bath application of CNQX (5 μM) and APV (50 μM). The IPSC were identified as mediated by $GABA_A$ receptors by their sensitivity to block by picrotoxin (50 μM) and comparing their experimental reversal potential with the calculated Cl^- reversal potential (-47 mV) (Jiang et al., 1997, Kim and Dudek, 1992). Picrotoxin, APV, and CNQX were obtained from Sigma (St. Louis, MO)

Application of test compounds

To study the presynaptic modulation of P/Q- and N-type Ca^{2+} channels by N/OFQ receptor activation, ω-agatoxin TK (500 nM), and ω-conotoxin GVIA (1 μM) (both Alamone Labs, Jerusalem, Israel), were used, respectively. ω-Agatoxin TK or ω-conotoxin GVIA were bath-applied for 20 min through a 100 μm diameter glass pipette placed close to the slice and aimed at the SCN. The ACSF containing the toxins flowed out of the perfusion pipette in the same direction and parallel to the flow of ACSF in the chamber. After the current stabilized during 15 min of toxin application, N/OFQ (1 μM) was added together with the toxin. The effect on current amplitude was measured as the ratio of the control EPSC amplitude to the EPSC amplitude recorded during toxin application. AMPA (100 μM) and NMDA (100 μM) were pressure ejected from a micropipette (2 μm tip diameter) located 30 – 50 μm from the recorded neuron using a Picospritzer (General Valve, Fairfield, NJ). [Nphe(1), Arg(14), Lys(15)]N/OFQ-NH(2) (UFP-101), an ORL1 receptor antagonist, was purchased from Tocris (Ellisville, MO).

Optical Ca^{2+} *measurements*

Ca^{2+} sensitive probes were prepared as a 2.5 mM stock of Fura-Red-AM, Fluo-5F AM or Oregon Green 488 BAPTA-5N-AM (OG-5N) (Molecular Probes, Eugene, OR) dissolved in DMSO, diluted with double strength ACSF (without Mg^{2+}, Ca^{2+} or PO_4^{2-}) to a final concentration of 250 μM and sonicated for 20 - 30 min. Glass pipettes containing this solution were used to slowly pressure inject (Picospritzer II, General Valve Corp., Fairfield, NJ) the Ca^{2+} probe into the optic chiasm approximately 250 – 300 μm away from the SCN. The pipette tip was positioned at an angle pointing away from the SCN. A suction pipette adjacent to the site of injection removed extraneous

probe. To further reduce the possibility of depositing probe outside of the optic chiasm, the flow (2 ml/min) of ACSF in the chamber was in a direction away from the SCN. Following injection, 2 - 3 hours were allowed for cleaving the ester and transport of probe into the RHT terminals in the SCN. A small amount of Texas Red-Dextran (Molecular Probes, Eugene, OR) was added to the injection solution in experiments using fluo-5F AM to help visualize probe loading. Fluorescent images were obtained with an upright microscope (DM LFS; Leica, Nussloch, Germany) with a water immersion objective (HCX AP L63X/0.9W U-V-I, Leica, Nussloch Germany). Excitation light from a monochromator (Polychrome II or IV; Till Photonics, Martinsried, Germany) with a bandwidth of 15 nm was attenuated with a 0.6 or 1.3 neutral density filter (Chroma, Brattleboro, VT), passed through a band-pass filter (Fura Red, D500/200; Fluo 5F and OG-5N, HQ480/40x, Chroma) and reflected by a dichroic mirror (Fura red 595dcxr; Fluo 5F and OG-5N, Q505LP; Chroma). The emitted light was filtered (Fura Red HQ645/75; Fluo 5F and OG-5N, HQ535/50m, Chroma) and recordings were made with either a photodiode (Till Photonics S4753-02, Martinsried, Germany) or a cooled charge-coupled device (CCD, 16 bit level) camera (C6790; Hamamatsu Photonics, Hamamatsu Japan) with binning at 32 x 32 to minimize photobleaching of the probe and maximize speed of data collection (3-49 msec/image). Photodiode experiments were controlled by Pulse software (HEKA, Lambrecht, Germany). Digital imaging software was used in experiments where images were captured a CCD camera (ARGUS HiSCA; Hamamatsu Photonics, Hamamatsu City, Japan).

During the recording, slices were placed in a 0.5 ml bath chamber (25°C) to increase stability of the probe. A perfusion device consisted of computer controlled

microsolinoid valves and a water-jacketed array of 8 tubes that emptied into a common tip positioned approximately 4 mm from the SCN at a flow rate of about 2 ml/min. Electrical stimulation was via a concentric bipolar electrode placed in the optic chiasm about 200 μm from the SCN providing 200 μsec pulses at 0.083 Hz (PG4000 Digital Stimulator and SIU90; Neurodata Instrument Corp, New York, NY). Voltage was adjusted to elicit a submaximal response. Optical data from photodiode and images from the CCD camera were converted to relative fluorescence intensity units (F), background was subtracted and the stimulation-induced presynaptic Ca^{2+} transients were measured using the fluorescence ratio $\Delta F / Fo$. Changes in resting Ca^{2+} were made using the high affinity probe Fura Red-AM alternating between two excitation wavelengths 488 nm and 465 nm. The latter wavelength was selected as the isosbestic point for Fura red from the Molecular Probes Inc. spectra. Changes in resting Ca^{2+} concentration were estimated by linearizing the probe response to Ca^{2+} using the formula (R-Rmin) / (Rmax-R), where R is the ratio of fluorescence (f) at f465 nm/f488 nm (Grynkiewicz et al., 1985). Rmin and Rmax were determined *in vitro* using the tetrapotassium salt of Fura Red under conditions with the buffer containing zero Ca^{2+} with EGTA (5 mM) and high Ca^{2+} (5 mM) respectively.

Statistical analysis

All data are presented as the mean ± sem, determined with a linear mixed-effects model using the statistical program 'R' (version 1.6.1 with nlme package version 3.1-3.6; obtained from http://cran.r-project.org; The R Foundation) (Ihaka and Gentleman, 1996). Additional statistical tests were performed with Statview (SAS Institute Inc., Cary, NC). A 95% confidence level determined statistical significance.

Results

N/OFQ inhibition of excitatory synaptic transmission

To examine whether N/OFQ modulates glutamate-mediated synaptic transmission of the RHT, excitatory postsynaptic currents (EPSCs) were evoked, in the presence of picrotoxin (50 μM), by a concentric bipolar stimulating electrode placed in the optic chiasm (Fig. 1A). N/OFQ (1-1000 nM) reduced, in a dose-dependent manner, the amplitude of EPSC recorded during daytime hours (35 of 42 cells). The IC_{50} was estimated by curve fitting using a logistic equation to be 10 nM with a maximal inhibition of 43.1 ± 4.0% (Fig. 10B, n = 5). No significant day-night difference was found in the N/OFQ-mediated EPSC inhibition at either 100 nM (76.3 ± 2.8% control EPSC amplitude night vs 74.4 ± 3.2% day, p = 0.74, n = 3) or 1 μM (55.6 ± 3.4 % control EPSC amplitude night vs. 56.9 ± 4, p = 0.59, n = 4, Fig. 10B). All remaining experiments were performed during daytime hours.

UFP-101 is a competitive antagonist of N/OFQ at its high affinity receptor, the ORL1 receptor (Calo et al., 2002, McDonald et al., 2003). Application of UFP-101 (1 μM) had no effect on the EPSC amplitude. However, UFP-101 (1 μM) completely prevented the reduction of the EPSC amplitude normally induced by N/OFQ (100 nM, Fig. 10C). The EPSC amplitudes in the presence of N/OFQ (100 nM) and UFP-101 (1 μM) were 94.7 ± 4.1% of control (p = 0.16, n = 5).

In contrast to the evoked EPSC, N/OFQ (300 nM), in the presence of 10 μM TTX and 50 μM picrotoxin (n = 4), did not alter either the mean mEPSC amplitude (15.3 ± 0.7 pA vs. 19.8 ± 3.3 pA, p = 0.3) or the mean rise time (2.7 ± 0.5 msec vs 1.9 ± 0.5 msec, p = 0.3). The lack of a N/OFQ effect on the mEPSC amplitude and the mEPSC kinetics demonstrates that N/OFQ is not altering the activity of postsynaptic

AMPA or NMDA receptors (Scanziani et al., 1992, Thompson et al., 1993). However, N/OFQ (300 nM) significantly reduced the frequency of miniature excitatory postsynaptic currents (mEPSC, 2.7 ± 0.8 Hz vs 0.4 ± 0.2 Hz, $p = 0.05$). The reduction of the mEPSC frequency but not amplitude or rise time is consistent with the conclusion that N/OFQ is acting on presynaptic receptors to reduce glutamate release from RHT terminals.

EPSCs evoked by optic nerve stimulation are mediated by both AMPA and NMDA receptors (Kim and Dudek, 1991, Jiang et al., 1997, Pennartz et al., 2001). NMDA (100 μM) or AMPA (100 μM) were focally applied to voltage-clamped SCN neurons. The amplitudes of the AMPA-activated currents in the presence of 300 nM N/OFQ were not significantly different from control (18.3 ± 2.1 pA control vs. 18.4 ± 2.7 pA N/OFQ, $p = 0.94$, $n = 5$, Fig. 10D). Similarly, N/OFQ (300 nM) had no effect on the NMDA-induced current amplitude (24.2 ± 7 pA control vs. 24.1 ± 6.2 pA N/OFQ, $p = 0.98$, $n = 6$, Fig. 1D). These data provide additional evidence to support the conclusion that N/OFQ does not modulate postsynaptic AMPA or NMDA receptors.

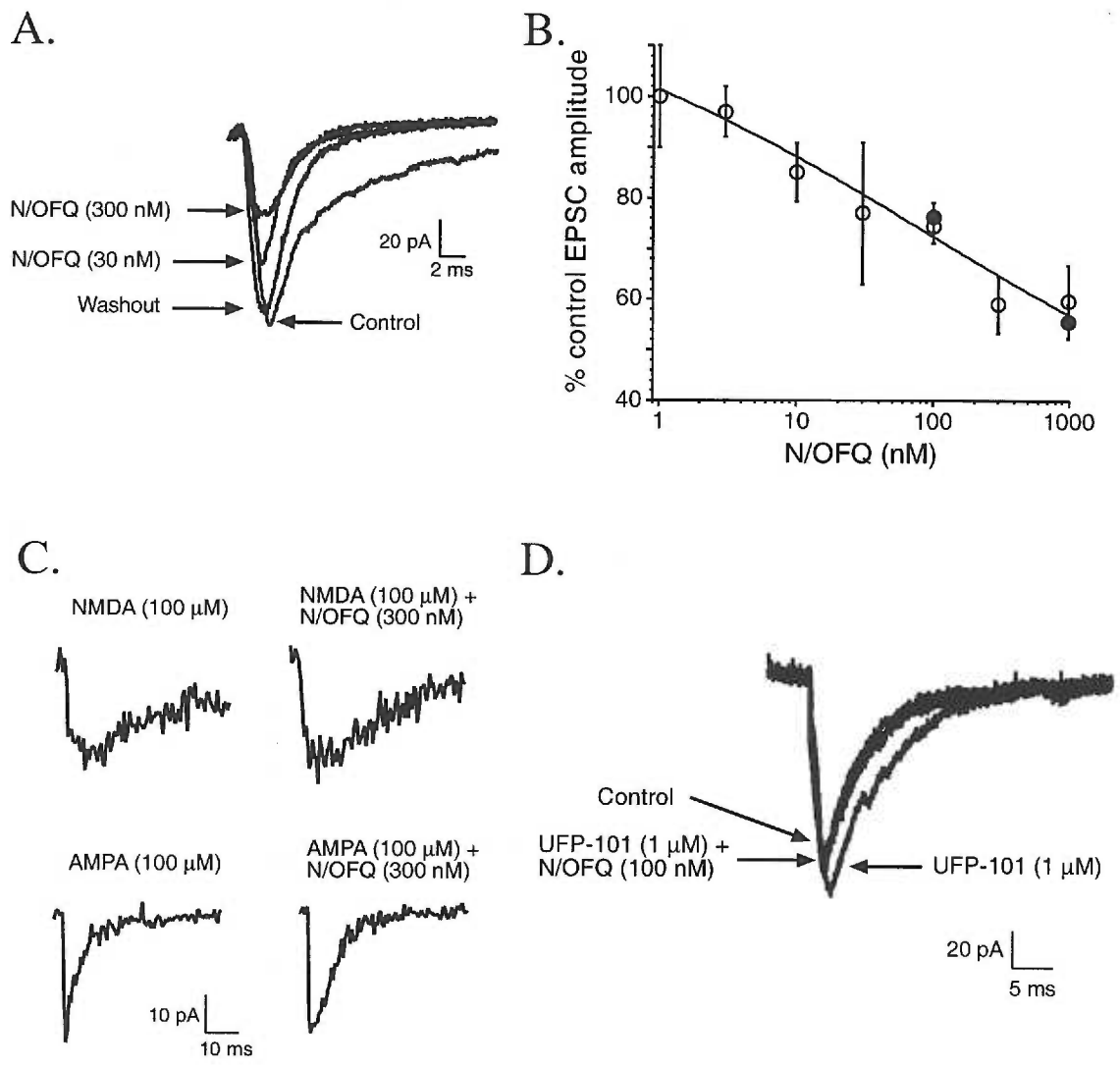

Figure 10. Effects of N/OFQ on the EPSCs recorded following RHT stimulation. A. Example of EPSCs evoked by optic nerve stimulation. Control current, EPSC in the presence of bath applied N/OFQ (30 nM), EPSC in the presence of bath-applied N/OFQ (300 nM), and an EPSC after a 30 min washout. B. Concentration-response relationship of the magnitude of N/OFQ inhibition of the EPSC amplitude. N/OFQ (1 – 1000 nM) was bath applied during during the subjective day (open circles) and 100 nM and 1 µM during the subjective night (closed circle). Each point represents the average percent EPSC amplitude following N/OFQ application relative to control (n = 3 – 13 each) ± SEM. C. Currents in response to NMDA (100 µM, top two traces) or AMPA (100 µM, bottom two traces) in the presence (right two traces) and absence (left two traces) of N/OFQ (300 nM). D. UFP-101 (1 µM) completely antagonized the reduction of the EPSC amplitude produced by N/OFQ (100 nM).

N/OFQ modulation of presynaptic Ca^{2+} channel activity

Experiments were performed to assess whether N/OFQ reduced the EPSC amplitude by inhibiting the P/Q or N-type Ca^{2+} channels involved in presynaptic RHT glutamate release. EPSCs were evoked as above while ω-agatoxin TK (500 nM), an inhibitor of P/Q-type Ca^{2+} channels, or ω-conotoxin GVIA (1 μM), an inhibitor of N-type Ca^{2+} channels, was applied directly onto the area surrounding the recorded cell using a glass micropipette. Following toxin application the EPSC recordings were continued until a steady state amplitude was reached. After the EPSC amplitude stabilized, 1 μM N/OFQ the concentration showing maximal effect in the dose-response curve, was applied to the recorded cell in the presence of the toxin. ω-agatoxin TK (500 nM) decreased the EPSC amplitude to 66.9 ± 7.5% of control (n = 8), and the addition of N/OFQ (1 μM) with ω-agatoxin TK reduced it further to 45.8 ± 6.7% of control (n = 8, p = 0.001, Fig. 11A). N/OFQ (1 μM) alone reduced the evoked EPSC amplitude to 67.3 ± 4.6% of control (n = 11). The 21.1% reduction produced by N/OFQ (1 μM) in the presence of ω-agatoxin TK is significantly smaller than the 32.7% reduction produced by N/OFQ alone (unpaired t-test, $p<0.015$). The observation that ω-agatoxin TK could reduce the N/OFQ-induced inhibition suggests that the N/OFQ effects are mediated in part by P/Q-type Ca^{2+} channels.

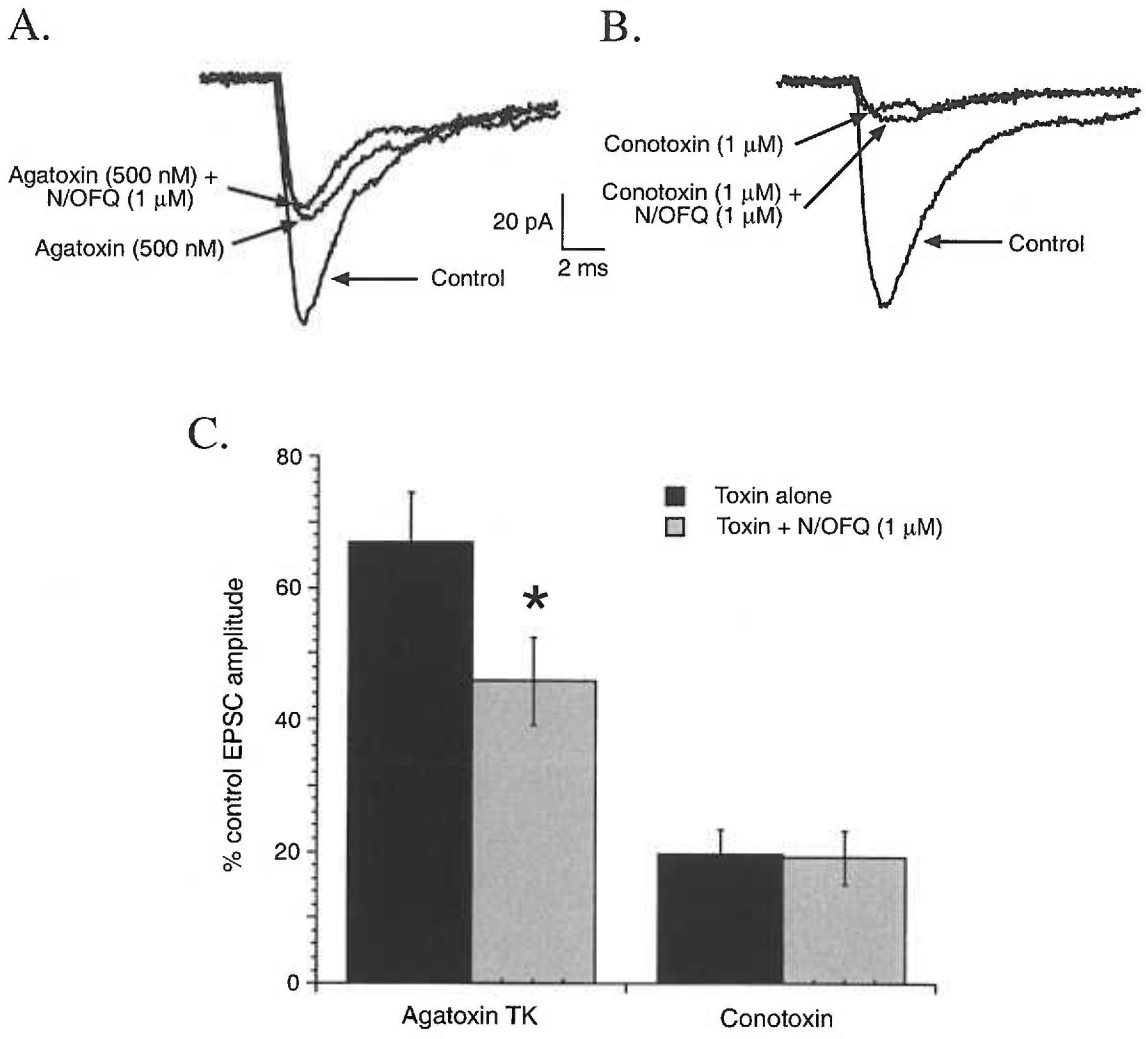

Figure 11. N/OFQ modulation of Ca^{2+} channel activity at RHT terminals. A, B. EPSCs in the absence and presence of toxin (A. w-agatoxin TK (500 nM), B. w-conotoxin GVIA (1 μM)) and toxin + N/OFQ (1 μM). C. The mean effect of w-agatoxin TK (n = 8) and w-conotoxin GVIA (n = 6) on EPSC amplitude were measured as the percent amplitude relative the EPSC amplitude recorded before toxin application. Additional inhibitory action of N/OFQ (1 μM) on EPSC amplitude occurred only in the presence of w-agatoxin TK, indicating that the inhibitory effect of N/OFQ on glutamate release is mediated largely by w-conotoxin GVIA-sensitive N-type voltage activated Ca^{2+} channels.

Application of ω-conotoxin GVIA (1 μM) reduced the EPSC amplitude to 19.7 ± 3.6% of control. N/OFQ (1 μM) produced no additional reduction in current amplitude (19.1 ± 4.1% of control, n = 7, p = 0.91, Fig. 11B). The inability of N/OFQ to further reduce the EPSC in the presence of ω-conotoxin GVIA suggests that N/OFQ is reducing glutamate release by inhibiting N-type Ca^{2+} channels. The complete block of N/OFQ's effect further suggests that the ORL1 receptor is tightly coupled to N-type Ca^{2+} channels and indicates that N/OFQ inhibits glutamate release at RHT terminals mainly by inhibiting presynaptic N-type voltage-activated Ca^{2+} channels.

Effect of N/OFQ on Ca^{2+} concentration in RHT terminals

N/OFQ reduced the magnitude of the evoked EPSCs in SCN neurons by inhibiting presynaptic Ca^{2+} channels. To evaluate if the Ca^{2+} channel inhibition attenuated the presynaptic Ca^{2+} transients that follow action potential depolarization, presynaptic RHT terminals were loaded with either Fura Red AM (high affinity Ca^{2+} probe), Fluo 5F AM (intermediate affinity) or Oregon Green 488 BAPTA 5N (low affinity). These probes exhibit an *in vitro* Ca^{2+} Kd of 0.14, 2.3 and 20 μM, respectively (Molecular Probes, Eugene, OR). Since the amplitude of Ca^{2+}-induced changes in fluorescence decrease with increasing Ca^{2+} concentration at sub-saturation levels, the three probes with varying Ca^{2+} affinities were used to measure a wide linear range of Ca^{2+} concentrations. Stimulation of the optic chiasm resulted in stepwise increases in the fluorescence ratio (ΔF/Fo) for all three probes tested (example Fluo 5F, Fig. 3A). The Ca^{2+} transients (example with Fura Red, Fig. 12B, n = 3) produced by repetitive pulsing were blocked by TTX (750 nM). Because presynaptic Ca^{2+} transients may saturate a high affinity Ca^{2+} probe, 4-aminopyridine (4-AP, 1 mM), which enhances

stimulation induced neurotransmitter release by acting as a K^+-channel blocker, was used to test for probe saturation. We found that 4-AP markedly enhanced transient Ca^{2+} signals produced by single electrical pulses confirming that under these experimental conditions our probe was not saturated (Fig. 12C).

The high affinity Ca^{2+} probe Fura Red AM was used to determine the effect of N/OFQ on the resting Ca^{2+} concentration in presynaptic RHT terminals. N/OFQ (300 nM) application lowered the presynaptic resting Ca^{2+} concentration (Fig. 12D). These experiments were performed in the presence of CNQX, APV, picrotoxin and CPG 55845 to block possible postsynaptic Ca^{2+} signals. The reduction in baseline Ca^{2+} levels was a consistent feature of all experiments. Calculating fluorescence ratios relative to the minimum (Rmin) and maximum (Rmax) ratios achieved in this preparation by using the equation [R-Rmin]/[Rmax-R], N/OFQ (300 nM) reduced the baseline Ca^{2+} concentration to 86.2 ± 0.9% of control ($p<0.0001$, n = 4 experiments). In contrast, no significant attenuation of electrically evoked RHT presynaptic Ca^{2+} transients was produced by N/OFQ in the presence of CNQX, APV, picrotoxin and CPG 55845 with any Ca^{2+} probes tested (Fig. 12E). Though not statistically significant, Fig. 3E shows a trend toward attenuated Ca^{2+} transients with the lower affinity probe, Oregon Green 488 BAPTA 5N (96.99 ± 1.55 %, baseline SEM: 1.38 %).

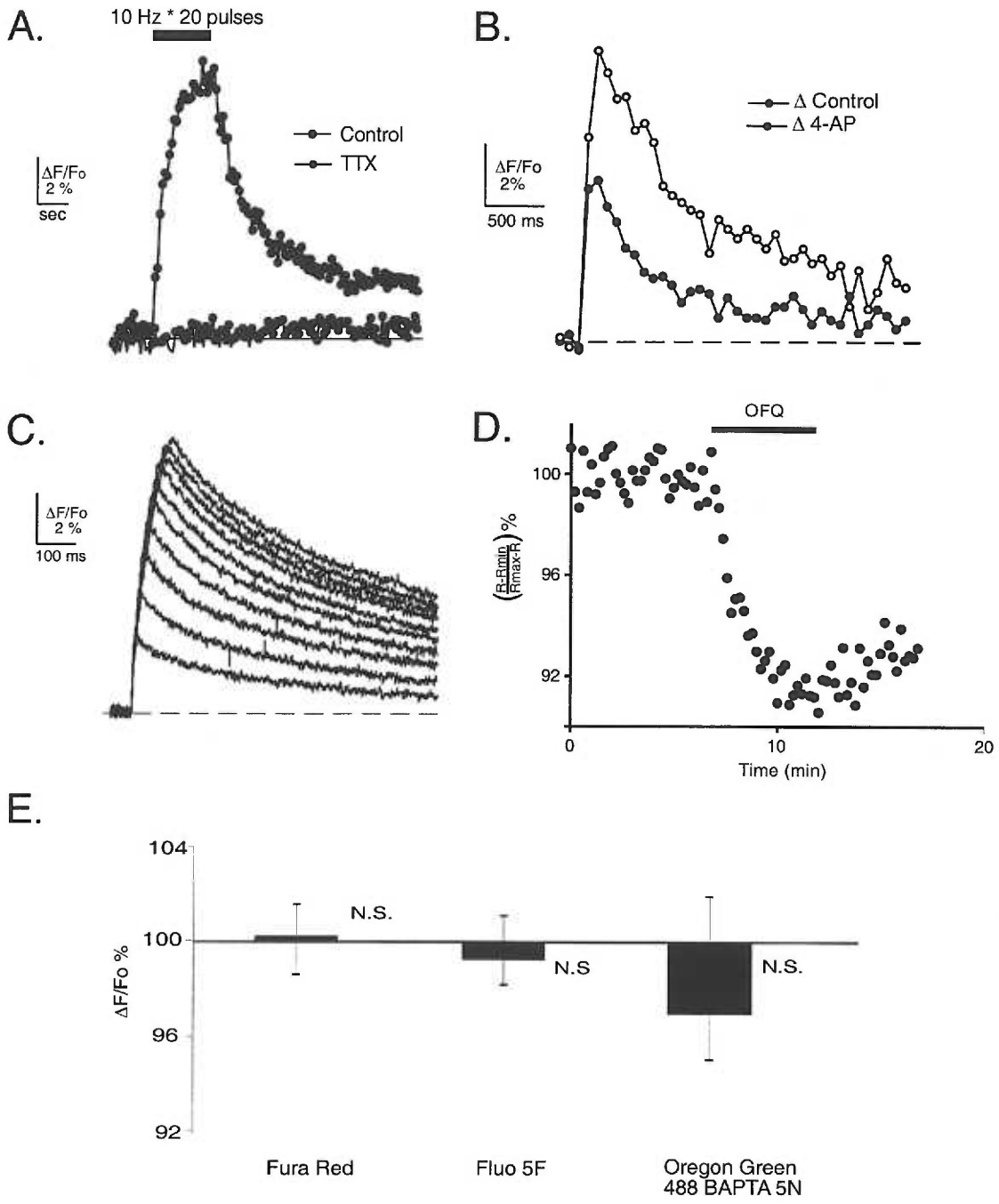

Figure 12. N/OFQ modulation of the presynaptic Ca2+ concentration. A. Ca2+ transients with repetitive stimulations (100 Hz). RHT terminals in the SCN were loaded with the Ca2+ sensitive probe Fluo5F AM (Kd ~ 2.3 μM). Each line represents the average of 5 pulses from one to ten stimulations. B. For optical recording in the SCN, the optic chiasm was loaded with the Ca2+ sensitive probe, Fura-Red (Kd ~ 0.14 μM) that was transported into RHT terminals. Presynaptic Ca2+ transients in RHT terminals were evoked by electrically stimulating (200 μsec) the optic chiasm. Sample tracing of the time course and optical ΔF/Fo Ca2+ transients measured in the SCN during conditions of control and TTX (750 nM, n = 3). C. Traces of the time course and optical ΔF/Fo Ca2+ transients from a single pulse during conditions of control and enhancement by 4-aminopyridine (4-AP, 1 mM, n = 3). Note that the Fura Red probe is not saturated by a single pulse. D. N/OFQ (300 nM) lowers resting presynaptic Ca2+ concentration. Example of a typical experiment using the high affinity Ca2+ sensitive probe Fura Red loaded in RHT terminals in the SCN. Each point was calculated using two excitation wavelengths. Example shown is R = 465 nm/488 nm. Rmin and Rmax were determined in vitro. E. N/OFQ did not significantly alter Ca2+ transients in RHT terminals in the SCN. Presynaptic RHT terminals loaded with Fura Red AM (n = 3), Fluo 5F AM (n = 2) or Oregon Green 488 BAPTA 5N AM (Kd ~20 μM, n = 7) were optically recorded at the SCN. Electrical stimulation at the optic chiasm was pulsed at a frequency of 0.083 Hz. Solutions contained (in μM): CNQX 10, APV 50, picrotoxin 50, and CGP55845 5. Bars represent the mean of N/OFQ (300 nM) treatment response from baseline response (normalized to 100%). For each experiment 15 Ca2+ transients were averaged for baseline and for N/OFQ. Error bars represent the SEM.

N/OFQ modulation of inhibitory synaptic transmission

$GABA_A$ receptor mediated inhibitory postsynaptic currents (IPSCs) were electrically evoked by a concentric bipolar stimulating electrode placed in the SCN and the effect of N/OFQ on absolute current amplitude examined (Fig. 13A). In the presence of APV (50 μM) and CNQX (5 μM), bath application of N/OFQ (3 -1000 nM) inhibited IPSC amplitudes (IC_{50} = 69 nM, maximal inhibition = 42.0 ± 3.8%) in 32 of 35 cells recorded during the day (Fig. 13B). The IPSC amplitude in the presence of N/OFQ (10 and 300 nM) relative to control for 7 cells recorded during the subjective night were not significantly different from daytime values (10 nM: 90.3 ± 5.1 % night vs. 90.9 ± 0.8 % day, p = 0.93 and 300 nM: 63.0 ± 11.0 % night vs. 63.9 ± 2.8 % day, p = 0.94). Inhibition of IPSCs by 100 nM N/OFQ was blocked by application of UFP-101 (1 μM, p = 0.2, n = 5, Fig. 13C).

To determine whether this action was mediated presynaptically or postsynaptically, we examined miniature IPSCs (mIPSC) in the presence of 10 μM TTX, 50 μM APV and 5 μM CNQX. While the addition of N/OFQ (300 nM) did not change the mIPSC amplitudes, the frequency was significantly reduced (3.9 ± 0.5 Hz in control to 1.8 ± 0.5 Hz in OFQ, p = 0.002, n = 6), as seen in the inter-event interval histogram from a representative cell (Fig. 13D). These data indicate that post-synaptic membrane modulation of the $GABA_A$ receptor-mediated current was not responsible for the decrease in current amplitude observed for the evoked IPSCs. The data also indicate that N/OFQ exerts part of its effect on GABA release directly on the release machinery (Scanziani et al., 1992, Thompson et al., 1993). The lack of postsynaptic involvement was supported by the observation that N/OFQ (300 nM) did not alter the amplitude of currents evoked by focal application of muscimol (100 μM, n = 7).

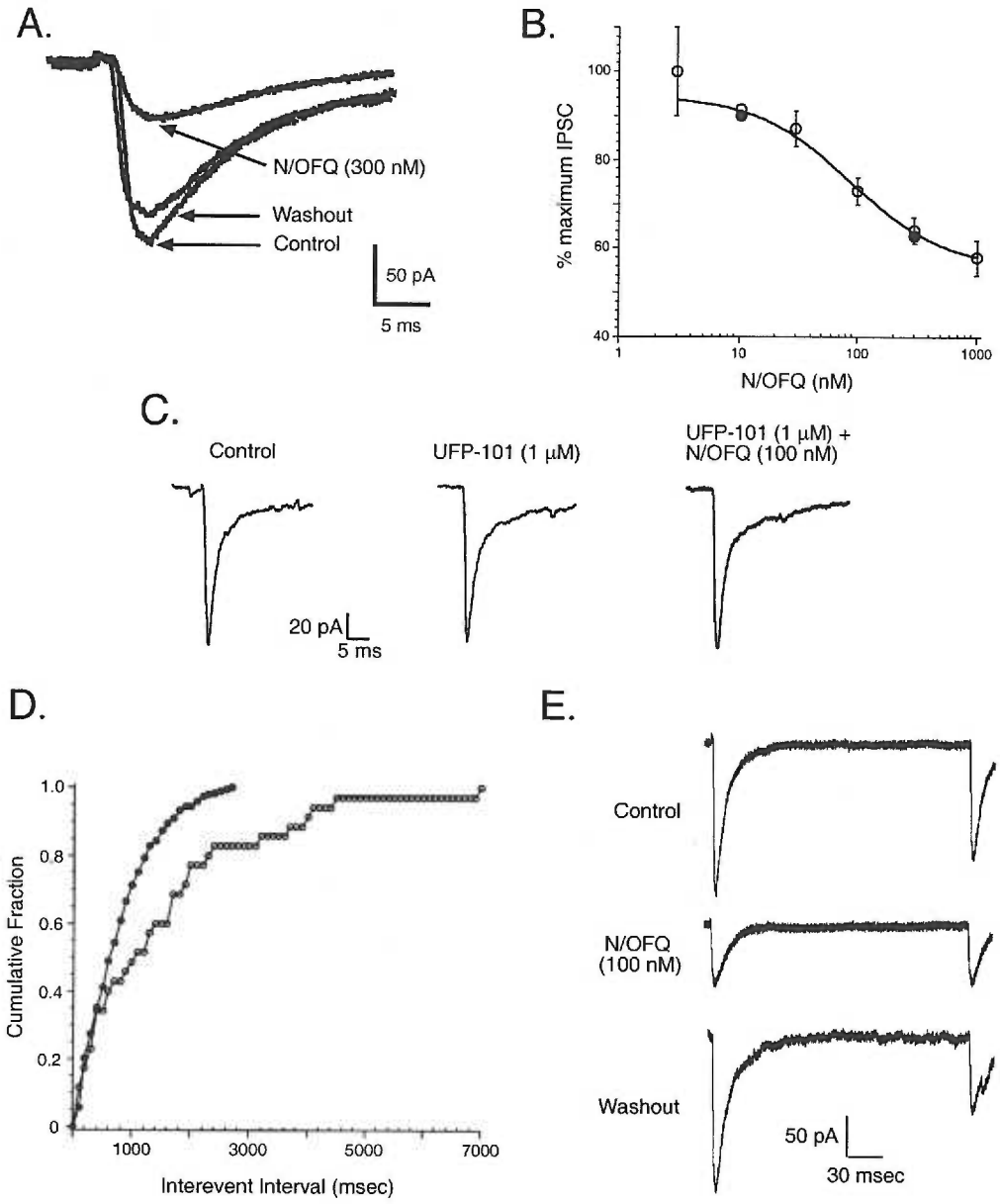

Figure 13. Effects of N/OFQ on GABAA receptor mediated IPSCs. A. Example evoked IPSC traces in control, 300 nM N/OFQ, and washout conditions. Currents were evoked by a concentric bipolar stimulating electrode placed within the SCN, in the presence of 50 μM APV, 5 μM CNQX. B. Dose response relationship of the inhibition of the IPSC amplitude produced by N/OFQ application. N/OFQ (3 -1000 nM) was bath applied during the day (open circles) and 30 or 300 nM during the night (closed circles) (n = 31). Each point represents the average percent IPSC amplitude relative to control (n = 3 – 9 cells each) ± SEM. C. UFP-101 completely blocked the reduction of the IPSC amplitude produced by N/OFQ application. D. Spontaneous IPSC inter-event interval histogram for a representative cell; a total of 6 cells were recorded that each showed similar histograms. Spontaneous, miniature IPSCs were recorded during 10 sweeps of 2 sec. duration (10 μM TTX, 50 μM APV, 5 μM CNQX). The reduction in IPSC frequency displayed in the presence of 300 nM N/OFQ (open circles) compared to control (closed circles) is consistent with a presynaptic site of action. E. Paired currents evoked by focal stimulation (200 msec interstimulus interval) of the SCN in the presence of CNQX (5 μM) and APV (50 μM). Note that the amplitude of the second IPSC is smaller than the first IPSC in the first and third traces. In the presence of N/OFQ (100 nM) the amplitude of the first IPSC is significantly reduced and the paired pulse ratio is near 1.

Application of paired stimuli to the SCN results in paired pulse depression mediated by a presynaptic mechanism in a subset of SCN neurons (Gompf and Allen, 2004). At an interstimulus interval of 200 msec, the ratio of the amplitude of the second IPSC to the amplitude of the first IPSC was 0.74 ± 0.04 (n = 5). N/OFQ (100 nM) eliminated the paired pulse depression producing a ratio of 1.1 ± 0.2 (Fig. 13E). These results indicate that N/OFQ, acting via presynaptic ORL1 receptors, consistently suppresses GABA release from SCN neurons.

Discussion

N/OFQ regulation of glutamate release

The glutamatergic RHT transmits changes in light intensity from the retina directly to SCN neurons, which acts to entrain the SCN's circadian rhythm with the evironmental light/dark cycle (Pickard, 1982, Berson et al., 2002). The light-induced phase shifts are proposed to be inhibited by activation of presynaptic 5-HT or $GABA_B$ receptors reducing glutamate release from RHT terminals (Jiang et al., 1995, Pickard et al., 1996, Pickard and Rea, 1997a, Pickard et al., 1999, Jiang et al., 2000). Similarly, N/OFQ can block light-induced phase advances when applied at CT19 (Allen et al., 1999). We therefore tested the hypothesis that N/OFQ acting on presynaptic receptors could inhibit the release of glutamate from RHT terminals. Since N/OFQ modulates the activity of most SCN neurons we also tested the hypothesis that N/OFQ could inhibit GABA release by acting at presynaptic ORL-1 receptors. The data presented here indicates that N/OFQ acting via ORL1 receptors inhibits glutamate release from presynaptic RHT terminals by a variety of mechanisms, including an inhibition of N- and P/Q-type Ca^{2+} channels, a reduction in the presynaptic baseline Ca^{2+} concentration

and an inhibition of the synaptic vesicle release machinery. Overall, these mechanisms contribute to the observed dose-dependent inhibition of EPSC amplitude (Fig. 1B). N/OFQ also inhibits the release of GABA from terminals in the SCN.

Examining the glutamatergic RHT-SCN synapse electrophysiologically, we found that N/OFQ dose-dependently inhibits glutamate-mediated currents in the SCN. However, N/OFQ had no effect on the AMPA- and NMDA-induced currents recorded from postsynaptic SCN neurons. One implication of these findings is that the N/OFQ effect on the EPSC amplitude is not due to a modulation of postsynaptic AMPA or NMDA receptor. Also, miniature EPSC frequency was reduced in the presence of N/OFQ while current rise time and amplitude were unaffected, further indicating a presynaptic site of action, and the possibility that N/OFQ inhibits the vesicular release machinery at RHT terminals.

Inhibition of presynaptic Ca^{2+} channels

In many neurons N/OFQ inhibits voltage activated Ca^{2+} channels (Beedle et al., 2004, Connor and Christie, 1998, Moran et al., 2000). Here, we examined the effects of N/OFQ on N- and P/Q-type channels at RHT terminals using specific blockers. ω-Conotoxin GVIA completly inhibited the N/OFQ mediated inhibition of EPSCs evoked by optic nerve stimulation. We interpret our inability to detect additional EPSC inhibition beyond that produced by the Ca^{2+} channel toxin to mean that the activation of presynaptic N-type Ca^{2+} channels is necessary for the N/OFQ induced inhibition. These data are consistent with observations from periaqueductal gray neurons that the N/OFQ receptor ORL1 specifically associates with and inhibits N-type channels (Beedle et al., 2004). ω-Agatoxin TK produced a small reduction in the ability of

N/OFQ to reduce the EPSC amplitude. This finding suggests that ORL1 receptors are also coupled to P/Q-type Ca^{2+} channels at RHT terminals but that the majority of the N/OFQ effect is due to inhibition of N-type Ca^{2+} channels. Thus our results demonstrate that N/OFQ acts largely by inhibiting presynaptic N-type channels at RHT terminals in addition to the effects on P/Q-type Ca^{2+} channels and the presynaptic release machinery downstream of Ca^{2+} entry.

To further characterize the effects of N/OFQ on Ca^{2+} levels in RHT terminals, we loaded RHT axon terminals with Ca^{2+} sensitive dyes and measured resting Ca^{2+} and evoked transients. Using the high-affinity ratiometric Ca^{2+} probe Fura-Red, resting Ca^{2+} levels were consistently lowered in response to N/OFQ application (Fig. 11D). Evoked presynaptic Ca^{2+} transients were measured using three different probes: Fura Red, Fluo 5F, and Oregon Green 488 BAPTA 5N. These Ca^{2+} probes respond in a near linear fashion at concentrations close to their Kd, and when used in conjunction provide quantitative results on Ca^{2+} levels from the nM to the μM range. Generally, the effect of N/OFQ on lowering Ca^{2+} transients was larger with lower affinity probes but did not reach statistical significance.

To capture fast Ca^{2+} transients, each probe was used non-ratiometrically. A possible source of underestimating the magnitude of the N/OFQ effect on stimulating Ca^{2+} transients could be caused by measuring the change in fluorescence ($\Delta F/Fo$) rather than directly quantifying the magnitude of the Ca^{2+} level. Calculating $\Delta F/Fo$ assumes that the resting fluorescence (Fo) has not changed. This study has demonstrated that N/OFQ exposure lowers resting presynaptic Ca^{2+}, thus while N/OFQ may not significantly change the relative ΔF, the absolute magnitude of the $[Ca^{2+}]$ increase in total may be lower in the terminal. It is conceivable that relatively small changes in peak

Ca^{2+} concentration in the terminals may have a large effect on the induction of glutamate release, as has been shown in other studies (Dittman and Regehr, 1996, Rosato-Siri et al., 2002). Another possible explanation for the lack of statistical significance is the inclusion of both responsive and non-responsive RHT terminals in these measurements.

N/OFQ inhibition of presynaptic GABA release

N/OFQ has previously been demonstrated to inhibit baseline Ca^{2+} levels and evoke K^+ currents in the vast majority of SCN neurons (Allen et al., 1999, Ikeda et al., 2003). We hypothesized that if these somatic actions translate to similar changes at axon terminals, an attenuation of neurotransmitter release between SCN neurons would be expected. The majority of SCN neurons make GABAergic synapses with other SCN neurons (Kim and Dudek, 1992, Jiang et al., 1997, Strecker et al., 1997), and GABA can phase shift the SCN (Ralph and Menaker, 1985). In this study, N/OFQ dose-dependently inhibited evoked $GABA_A$ receptor mediated IPSC currents in the majority of measured cells. However, N/OFQ had no effect on currents activated by muscimol, a $GABA_A$ agonist, or on the amplitude of the mIPSC. These data demonstrate that N/OFQ is not acting to modulate the activity of postsynaptic $GABA_A$ receptors. N/OFQ significantly reduced the mIPSC frequency consistent with an inhibition of GABA release downstream from Ca^{2+} entry. Finally, N/OFQ inhibited the paired pulse depression observed in SCN GABAergic synapses consistent with a presynaptic mechanism of action. $GABA_A$ receptor activation can directly phase shift the circadian clock and also attenuate glutamate mediated light-induced phase shifts (Ralph and Menaker, 1985, Mintz et al., 2002). Thus, the result of GABAergic synaptic modulation by N/OFQ on phase-shifting stimuli in the SCN may depend upon the time

context at which the peptide is released *in vivo*.

Conclusion

Ongoing stability in neural systems depends in part on the dynamic reweighing of synaptic inputs. In this paper we have described a role for the neuromodulator N/OFQ in reversibly reducing the amount of neurotransmitter released from excitatory and inhibitory synapses. N/OFQ reduces, but does not completely abolish glutamate release from RHT terminals. This effect is mediated by a combination of presynaptic mechanisms, including inhibition of N-type and P/Q-type voltage activated Ca^{2+} channels, a reduction in baseline Ca^{2+} levels, and an inhibition of the presynaptic release machinery. N/OFQ also reduces, but does not completely block GABA release at intra-SCN synapses. This effect is mediated by an inhibition of the presynaptic release machinery, while the previously described reduction in baseline Ca^{2+} levels and stimulation of the K^{+}-current may also contribute to this effect. Numerous other peptides, such as PACAP (Kopp et al., 2001, Harmar, 2003), VIP (Huang and Pan, 1993, Shibata et al., 1994, Gillespie et al., 1996), or NPY (Obrietan and van den Pol, 1996, Biello et al., 1997, Yannielli and Harrington, 2001) have been shown to influence SCN synaptic transmission. While PACAP and VIP have been shown to enhance glutamatergic and GABAergic neurotransmission in the SCN, respectively, a peptide neurotransmitter with such widespread effects in the SCN has thus far not been described.

Acknowledgements

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IV. Discussion and Conclusions

Synaptic communication in the SCN is integrally involved in the synchronization of the endogenous cellular rhythms with the environmental light/dark cycle as well as the coupling between SCN neurons to produce a coherent circadian output signal to the organism. The work presented here demonstrates that synaptic transmission is influenced by the action potential firing frequencies of both the presynaptic and postsynaptic cells, by the time of day and by the inhibitory neuropeptide N/OFQ. These influences presumably modulate the fast synaptic transmission in the SCN so that the information conveyed is relevant to the longer 24-hour circadian cycle.

Whole-cell brain slice electrophysiology was used throughout this study to demonstrate these modulatory mechanisms at SCN synapses. Manuscript #1 showed that the synaptic strength of the GABAergic network in the SCN is modulated over the circadian cycle. Paired GABA-mediated IPSCs evoked in a range of frequencies relevant to SCN physiology were more likely to exhibit paired-pulse depression during the early daytime hours than later in the day, while paired-pulse depression was rarely found during the subjective night. Manuscript #2 describes a form of synaptic inhibition in which the postsynaptic cell directly inhibits presynaptic GABA release via the release of a retrograde signal. Thus, the subset of neurons in the SCN capable of retrograde inhibition can block specific inhibitory inputs during times of neuronal excitability. Manuscript #3 shows the inhibitory effects of the neuromodulatory peptide N/OFQ on neurotransmitter release in the SCN. The widespread effects of this peptide indicate its function as an important neuromodulatory peptide in the SCN.

The SCN as a circuit of inhibitory GABA cells

The circadian pacemaker in mammals is the suprachiasmatic nucleus of the hypothalamus, which is composed of multiple cellular oscillators that couple, to loosely synchronize each other's rhythms into a coherent day / night rhythm. Several cellular communication mechanisms have been proposed for this synchronization between the individual oscillators, including calcium spikes (Bouskila and Dudek, 1993), electrical coupling (Jiang, Yang, and Allen, 1997b), and paracrine or ionic interactions (van den Pol and Dudek, 1993). However, synaptic mechanisms of synchronization have also been observed. Specifically in slice culture, cell pairs whose action potential firing frequencies are directly coupled have been shown to depend on GABA-ergic synapses for this synchronizing mechanism (Honma et al., 2000; Shirakawa et al., 2000). These data provide additional mechanistic evidence to observations showing that SCN cells synchronize neuronal activity in response to $GABA_A$ receptor agonists (Liu and Reppert, 2000). These studies were performed using SCN slice cultures grown on multi-electrode array dishes (MEAD) and recorded ensemble and individual action potential firing frequencies. While the latter study used long-term exposures of high doses of GABA (100 μM for 3 hours) to demonstrate global synchronization of autonomous oscillators by $GABA_A$-mediated mechanisms, the former demonstrates that spontaneous $GABA_A$-mediated IPSCs synchronize individual pairs of neurons. The question, then, is how does this occur?

We know from *in vivo* (Schwartz et al., 1987) and *in vitro* (Earnest et al., 1991; Newman et al., 1992; Shibata and Moore, 1993) studies that synaptic communication can be completely blocked by TTX in the SCN for a matter of days, and the system is then able to re-establish the expected circadian rhythm after removal of the blockade of

synaptic communication. The circadian rhythm in the likelihood of cellular action potential firing also is restored to the point on the phase according to its expected circadian time. Hence, the intrinsic molecular clock probably determines the likelihood of an SCN neuron firing action potentials. However, the action potential firing pattern or whether two individual neurons fire synchronized action potentials may be determined by the direct synaptic interaction between neuron pairs. Evidence that $GABA_A$-mediated synchronization is a highly localized event (Honma et al., 2000; Shirakawa et al., 2000) points to the possibility that numerous such synchronizing connections can exist in a network of 8,000 cells, thereby providing a constant level of synchronizing inputs from cell to cell at the level of the synapse.

Exactly what sort of information is transmitted by these synapses? And, how can a network of presumably inhibitory synapses not impede the apparent increase in ensemble neuronal excitability during times of greater synaptic communication? While it has been questioned whether increased daytime ensemble firing rates lead to an increase in inhibitory transmission (Lundkvist, Kristensson, and Hill, 2002), IPSC frequency in the dorsal SCN has been found to be greater during the subjective night (Itri and Colwell, 2003), which would be exactly opposite of what is expected, given the circadian rhythm in action potential firing frequency. Still other researchers have found that GABA is an excitatory neurotransmitter during the subjective day and an inhibitory neurotransmitter during the subjective night (Wagner et al., 1997). However, this finding has been highly controversial and numerous other findings refute this assertion (Gribkoff et al., 1999; Gribkoff, Pieschl, and Dudek, 2003). One of the more recent studies has provided more conclusive evidence to refute the previous findings by showing that blocking $GABA_A$-mediated synaptic transmission in the SCN increases

firing frequency and changes its pattern from irregular to regular (Kononenko and Dudek, 2004), suggesting that GABA in the SCN is an inhibitory transmitter that communicates information to influence the firing patterns of neighboring cells. In light of my findings, the modulation of synaptic transmission at intra-SCN synapses is significant regardless of whether GABA is acting as an excitatory or an inhibitory neurotransmitter. However, since the exact mechanism of how GABA can act as an excitatory neurotransmitter has not been elucidated, my assumption during these experiments has been that GABA acting of the $GABA_A$ receptor is an inhibitory signal in intra-SCN synaptic transmission. Given that the SCN is a dynamic system coordinating the individual circadian oscillations of numerous cells and environmental and other inputs into a coherent temporal output, one might expect that modulating inhibitory synaptic neurotransmission plays an important role in shaping the quality of inter-neuronal communication. Therefore this series of studies was performed to determine some of the available modulatory mechanisms in the SCN.

Short-term synaptic plasticity in the GABAergic SCN network

To examine this issue, I evoked pairs of intra-SCN $GABA_A$-mediated IPSCs with inter-stimulus intervals that represent a large range of possible action potential firing frequencies in the SCN. The hypothesis in this set of experiments was that since GABAergic synaptic transmission is almost ubiquitous amongst SCN neurons and may play a role in shaping the circadian rhythm of action potential firing, modulation of this synaptic transmission via synaptic plasticity might play a prominent role in intra-SCN cell communication. Short-term synaptic plasticity—the inhibition of facilitation of synaptic neurotransmission that occurs over a matter of milliseconds—in the SCN

could occur over both a range of frequencies and a range of circadian times. Measuring pairs of currents evoked from neighboring SCN cells, I found a significant paired-pulse depression over a range of frequencies and diurnal times, that is mediated by a presynaptic site of action at the axon terminal.

Short-term synaptic plasticity as seen in this case indicates a low-pass filter that inhibits synaptic transmission at (PPD Types 1 and 2, Manuscript #1) or above (Type 1) a presynaptic action potential firing frequency of 5 Hz. While mechanistic distinctions between the two types of short-term synaptic plasticity was found, it is likely that Type 1 synapses actually express the same inhibition that type 2 synapses express at the 200 msec inter-stimulus interval and an additional plasticity at the 25 msec inter-stimulus interval. The overriding observation, however, that short-term plasticity is primarily a daytime event implies that synaptic transmission is regulated by the circadian clock and may represent part of an inter-cellular code at intra-SCN synapses. Such coding of synaptic transmission could help shape the degree or quality of coupling information relevant to intercellular synchronization of circadian rhythms over the course of the diurnal cycle. In dispersed SCN cell cultures, approximately 36% of neurons establish functional synaptic pairing, and those pairs have precisely synchronized circadian rhythms of action potential firing frequency (Shirakawa et al., 2000). This synchronization depends upon temporally correlated spikes in the neuron pairs that are the result of synaptic transmission, and neuron pairs can be de-synchronized following prolonged inhibition of synaptic transmission due to tetrodotoxin exposure(Honma et al., 2000). The intra-SCN synaptic transmission in these studies appeared to be excitatory during the active period, though the majority of

neurons in culture were positive for the inhibitory neurotransmitter GABA (Shirakawa et al., 2000), a result that is similar to SCN neurons in the acute brain slice preparation.

Usually when GABA activates $GABA_A$ receptors, as in the SCN, it acts as an inhibitory neurotransmitter. This is because the $GABA_A$ channel is permissive to Cl^- ions that under normal conditions flow into the cell and thereby act to hyperpolarize the cell membrane. During development, however, GABA has been shown to act as an excitatory neurotransmitter (Michelson and Wong, 1991; Chen et al., 1996), a result that can be explained by a shift in intracellular Cl^- concentration. Similarly, it was found that $GABA_A$-mediated neurotransmission is inhibitory during the subjective night and excitatory during the subjective day (Wagner et al., 1997). However, this has been challenged numerous times and it appears that the overwhelming majority of data do not support such a mechanism (Gribkoff et al., 1999). Alternatively, speculation that GABA-mediated neurotransmission leads to rebound action potentials has raised the possibility that the hyperpolarization-activated current ("H-current", or I_h) may be involved in this process in the SCN (Dudek et al., 1993; Jiang et al., 1997a). In other words, if the hyperpolarization provided by GABA activates the I_h, this would lead to a depolarization of the neuron above the resting membrane potential and induce a rebound action potential. However, it has been shown that I_h is relatively small in the SCN, and blocking it does not change either the action potential wave form or spontaneous action potential firing frequency (de Jeu and Pennartz, 1997).

Recent evidence from mathematical modeling studies indicates that short-term synaptic depression can lead to stable synchronization between neuron pairs. In inhibitory networks consisting of a pacemaker neuron receiving feedback from a follower neuron, the model suggests that synchronization is achieved using short-term

depression even when postsynaptic rebound properties (such as I_h) are weak or absent (Manor and Nadim, 2001). We also know from a recent study that $GABA_A$-mediated inputs can change the firing patterns of SCN cells from regular to irregular (Kononenko and Dudek, 2004). Synaptic depression during the early day could inhibit the ability of the presynaptic cell to influence postsynaptic firing, thereby favoring greater numbers of SCN neurons firing in a regular pattern. Later in the day or during the night, irregular firing could be favored because the strength of synaptic transmission is greater (due to the reduced likelihood of short-term inhibition) and this would tend to influence the postsynaptic voltage more than during time of short-term inhibition. In this way, synaptic depression might function as a signal that regulates intra-SCN synchronization by influencing postsynaptic firing patterns, and these patterns can also shape the longer-term circadian rise and fall of action potential firing rate or the synchronization between pairs of neurons over the thousands of neuron pairs in the circuit. A network of synchronized neurons are generally thought to provide a stronger output signal than desynchronized cells, and thus, the early portion of the daytime may be more conducive for the output of particular types of signals that require greater inter-neuronal synchrony.

Retrograde inhibition in the GABAergic network of the SCN

Another regulatory mechanism that was explored in this work was the rapid, direct inhibition of intra-SCN neurotransmission termed retrograde inhibition. In other brain regions, such as the hippocampus and the cerebellum, retrograde signaling has been shown to provide inhibition that generally lasts longer than the short-term plasticity described above (Pitler and Alger, 1994; Kreitzer and Regehr, 2001; Alger, 2002). Also,

retrograde inhibition allows synapse specificity, so that a neuron receiving multiple inputs can selectively inhibit some synapses and not others. In the SCN, it was previously found that a subpopulation of SCN neurons expresses dendritic dense-core vesicles, indicating the possibility of dendritic neuromodulator release in the SCN (Castel et al., 1996). Based partially on this finding, the hypothesis tested here was whether a subset of SCN neurons uses retrograde inhibition as a modulatory mechanism at the intra-SCN cell synapse. In the findings detailed in Manuscript #2, evoked and spontaneous GABBAergic currents were attenuated following postsynaptic depolarization in a subset of SCN neurons examined. This inhibition was also observed when examining evoked GABAergic currents during the day (40%) as well as the night (29%) and when the postsynaptic cell was depolarized at frequencies of 5 Hz. Retrograde inhibition was shown to depend upon postsynaptic Ca^{2+} influx and a presynaptic pertussis-toxin-sensitive G-protein coupled receptor. In conclusion, a small proportion of SCN cells is expected to inhibit specific synaptic inputs at the observed range of daytime spontaneous action potential firing frequencies.

From other brain areas, we know that retrograde inhibition requires significant Ca^{2+} influx (Lenz and Alger, 1999; Glitsch, Parra, and Llano, 2000; Brenowitz and Regehr, 2003). This makes the mechanism highly synapse-specific since this can often only be achieved in very localized portions of the dendritic tree. The results in Manuscript #2 also show a dependence upon elevated intracellular Ca^{2+}, and only a slight day/night variation was found in the likelihood of observing depolarization-induced inhibition. However, several factors could allow this mechanism to be more likely observed during the day, even if the whole-cell voltage clamp experiments could not resolve this difference due to the dilution of the intracellular environment. First, the

depolarization-induced inhibition was shown to be at least partially dependent upon Ca^{2+} influx through L-type channels. These channels have been shown to be under the control of the circadian clock such that greater Ca^{2+} influx is expected during the subjective day, an observation that implies a mechanistic explanation for the observed increase in daytime action potential firing frequency (Pennartz et al., 2002). This implies that the same channels partially responsible for the increase in action potential firing frequency during the subjective day in the SCN could also increase the likelihood of observing depolarization-induced inhibition at these firing frequencies. Secondly, we have recently found a circadian rhythm in baseline Ca^{2+} levels that peak in the early subjective daytime (around CT4) at roughly 400 nM (Ikeda et al., 2003). My hypothesis is that the retrograde messenger that brings about depolarization-induced inhibition in the SCN is packaged into the "dense core" vesicles previously described (Castel, Morris, and Belenky, 1996), and thus, an increase in intracellular $[Ca^{2+}]$ of this magnitude alone is unlikely to cause vesicular release. However, the increased baseline Ca^{2+} levels might have a significant impact on the probability that a given action potential could raise intracellular $[Ca^{2+}]$ levels to a point that allows vesicle fusion and release. Another alternative is that this $[Ca^{2+}]$ rhythm may affect the number of action potentials required to elicit retrograde messenger release. The dense core vesicles that presumably contain the retrograde messenger are located on the soma, but primarily on dendrites. From previous unpublished results, I have shown that changes in baseline Ca^{2+} levels—in this case a reduction in response to N/OFQ—are analogous in dendrites and soma. So, while the circadian rhythm in Ca^{2+} levels was shown in the soma, one could reasonably expect an analogous change in the dendrite. Thus, dense core vesicles

in the soma as well as in the dendrites could be more or less likely to release a putative retrograde messenger due to the Ca^{2+} rhythm.

One presently unanswered question is the identity of the retrograde messenger in the SCN. A commonly identified retrograde messenger in other brain regions has been endocannabinoids (Alger, 2002). Endocannabinoids are made on demand through cleavage of membrane precursors and are highly hydrophobic. Though it is presently unclear how they cross the synaptic cleft, it is known that vesicular packaging is not a factor in this signaling (Piomelli, 2003). The synthesis and release of a highly lipophilic messenger that diffuses from specific dendritic compartments is to a certain extent a more localized event in cells in the hippocampus, amygdala, or cerebellum than it might be in the SCN. In neurons of these areas the size, spatial arrangement, and dendritic arborization are quite different from the SCN where neurons tend to be tightly packed and small (~10 μm dia.), with relatively simple dendritic arborizations (Van den Pol, 1980;Guldner, 1984). Thus, endocannabinoids acting at a synapse on a specific portion of the dendrite are often viewed as "localized" events whereas the same probably cannot be said about these modulators if they were to be present in the SCN where their release could conceivably affect numerous neighboring neurons. In other words, what could be considered a relatively localized neurotransmission in other brain areas could be more global—and therefore less specific—in an area such as the SCN. Alternatively, the hypothesis that retrograde inhibition might modulate neurotransmission in the SCN is based in part on evidence showing that "dense-core vesicles" are present on dendrites in the SCN (Castel, Morris, and Belenky, 1996). Dense core vesicles often contain peptidergic, as opposed to lypophilic cannabionoid, neurotransmitters, and their presence on dendrites suggests a non-synaptic neuromodulatory role. Similar non-

endocannabinoid dendritic vesicular release has been found in the hypothalamus (Ludwig and Pittman, 2003). For instance, magnocellular neurons release the peptides vasopressin and oxytocin from their somato-dendritic compartments in the supraoptic and paraventricular nuclei. In this case, the peptides are responsible for a type of autoregulatory control of the cells they are released from, whereas the inhibition that I have observed appears to be a retrograde signaling event.

Any number of transmitters and modulators could potentially play a role in this mechanism in the SCN, including GABA itself acting on presynaptic $GABA_B$ receptors (Jiang, Allen, and North, 1995). Peptides synthesized by SCN neurons, including vasoactive intestinal protein (VIP), arginine-vasopressin, somatostatin, and gastrin-releasing peptide (GRP), could possibly play a role in modulating intra-SCN neurotransmission (van den Pol and Tsujimoto, 1985). However, vasopressin tends to increase SCN neuronal excitability (Ingram et al., 1998) and VIP enhances GABAergic neurotransmission (Harmar, 2003), making these two peptides unlikely inhibitory retrograde messengers. On the other hand, the inhibitory somatostatin receptors SSTR1 and 2 are expressed in a subpopulation of SCN neurons (Beaudet et al., 1995). As discussed below, N/OFQ is an inhibitory peptide at the majority of cells in the SCN. While no prepro N/OFQ mRNA expression has as yet been identified in the SCN, its expression may be temporally limited, thus requiring more expansive techniques to identify it in the SCN. Either way, the identification of the retrograde inhibitor in the SCN will require future study. However, identification of the retrograde transmitter could provide insight into the role these endogenous modulatory neuropeptides play in particular cell types. For instance, if the transmitter is found primarily in SCN projection neurons, it would suggest that this type of neuron inhibits synaptic inputs

during times of increased regular firing and could provide information about the type of synaptic output from the SCN.

The role of the modulatory peptide N/OFQ

Previous studies have shown that N/OFQ inhibits light-induced phase shifts but does not alter free-running circadian rhythms when microinjected into the hamster SCN (Allen et al., 1999). Additionally, N/OFQ dose-dependently evokes a K^+-current and lowers baseline Ca^{2+} levels in the vast majority of SCN neurons. These observations led to the hypothesis that N/OFQ may influence both excitatory and inhibitory synaptic transmission in the SCN. In Manuscript #3, it is shown that N/OFQ inhibits both GABAergic as well as glutamatergic synaptic transmission presynaptically during both daytime and nighttime recordings, an effect that was blocked by the ORL1-specific receptor antagonist, UFP-101 (Calo et al., 2003). This manuscript also reveals that N/OFQ inhibits presynaptic N-type Ca^{2+} channels, consistent with other observations about the N/OFQ receptor ORL1 (Beedle et al., 2004), and lowers baseline Ca^{2+} levels at RHT terminals. The data indicating presynaptic RHT inhibition are consistent with other data showing retinal cell expression of the N/OFQ receptor (Neal et al., 1997; Makman and Dvorkin, 1997) and provides detailed insight into the actions of this receptor at ganglion cell axon terminals in the SCN. Together, these data indicate a major role for this peptide in the modulation of SCN function.

The activity of N/OFQ in the SCN is clearly inhibitory, which stands in contrast to other peptides—notably PACAP and VIP—that have been shown to facilitate synaptic transmission in the SCN (Huang and Pan, 1993; Shibata et al., 1994a; Gillespie et al., 1996; Gillespie et al., 1997; Kopp, et al., 2001; Itri and Colwell, 2003;

Harmar, 2003; Itri et al., 2004). The previously observed effect on inhibiting light-induced phase shifts can reasonably be attributed to the inhibition of glutamate and GABA release. This study used the novel technique of loading presynaptic RHT terminals with Ca^{2+}-sensitive dyes to examine baseline levels, as well as evoked transients in RHT terminals. Thus far, only few other examples of retinal afferent terminal Ca^{2+} levels exist (Chen and Regehr, 2003). The combination of this technique along with electrophysiological recordings concluded that the combination of what can be seen as only a statistically insignificant reduction in evoked Ca^{2+} transients when measured using Ca^{2+} fluorescence is mediated through the inhibition of N-type voltage-activated Ca^{2+} channels. This reduction, along with the reduction in presynaptic baseline Ca^{2+} levels presumably results in the inhibition of glutamatergic currents measured postsynaptically. Based on other research demonstrating the involvement of GABAergic communication in the light-induced phase shift (Mintz et al., 2002), I conclude that the inhibition of light-induced phase shift advances from the combination of the inhibition of both glutamatergic and GABAergic neurotransmission in the SCN.

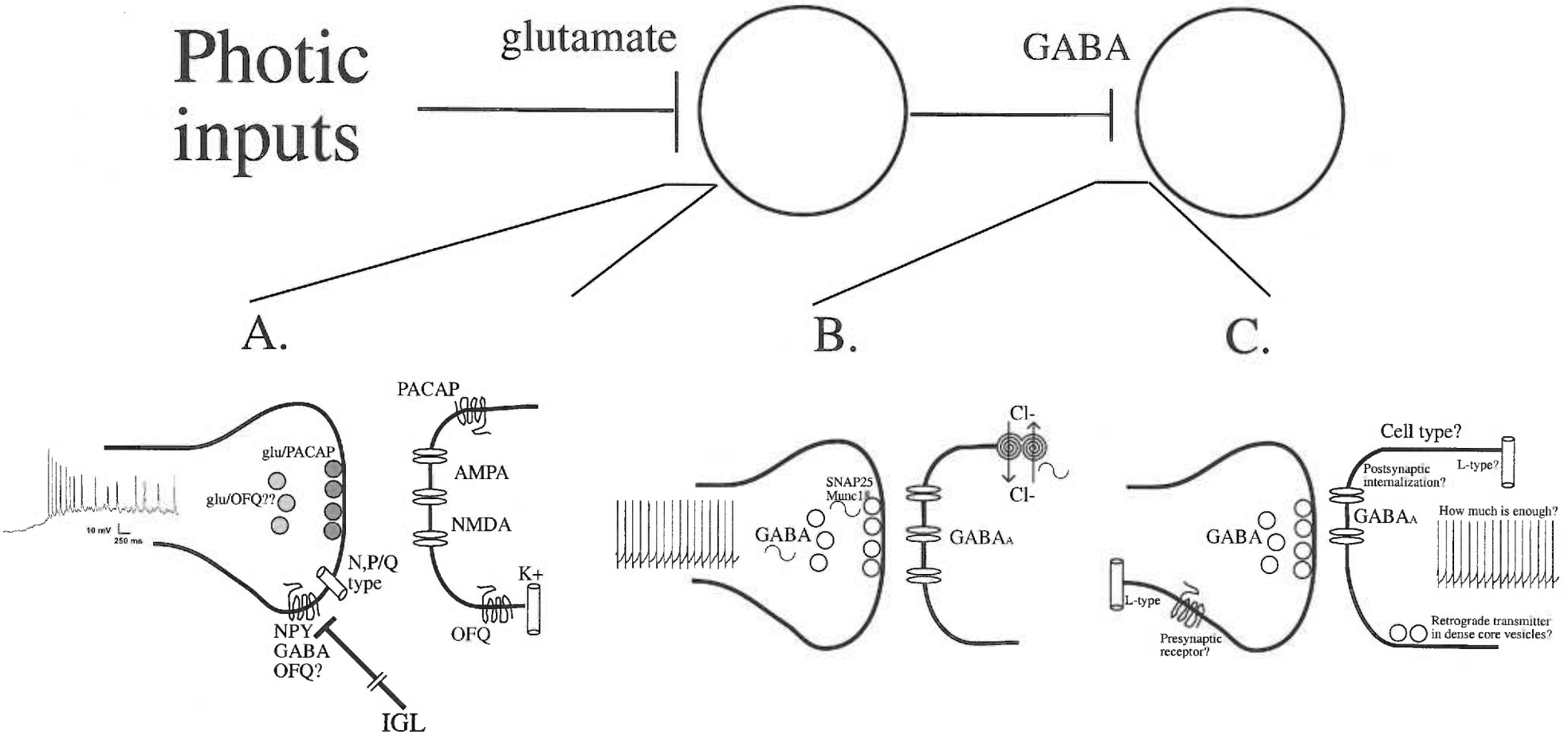

Figure 14: Schematic representation of a simplified circuit diagram of the SCN. A. Glutamatergic RHT input B., C. Intra-SCN GABAergic synapse. See text for detailed didscussion.

Synopsis and Future Directions:

A fairly detailed knowledge about the molecular mechanisms governing the expression of circadian rhythms in individual clock neurons of the SCN currently exists. How these molecular rhythms are translated into neuronal function, however, has not been examined in quite as much detail. Two of the most prominent features of the SCN are the extensive synaptic connections between SCN neurons and the strong synaptic inputs from the retina. The concept that synaptic transmission functions as the conduit between the molecular clock and the outside world led me to propose that modulatory mechanisms at the synapses of SCN neurons exist to allow for the integration of circadian temporal information between the individual oscillators. The studies presented in this thesis have allowed a detailed view into synaptic transmission in the SCN, and yet have also opened a number of further questions.

Figure 14 illustrates the concepts behind synaptic contribution to the cellular integration of molecular clocks in a schematic form of the simplest SCN circuit diagram. The circuit shows the glutamatergic retinal inputs as well as one intra-SCN synapse. Figure 14A is a detailed view of some of the issues discussed here surrounding the glutamatergic inputs from the RHT. It shows a presynaptic RHT axon terminal with the firing pattern of an SCN-projecting retinal ganglion cell recorded at the cell body (trace provided by Erin Warren). Of note is the rapid reduction in action potential firing frequency following initial light exposure. As mentioned in the Introduction, if this trace accurately represents glutamate-release promoting action potentials at the axon terminals, neuromodulatory factors at the RHT-SCN synapse would most likely need to be present during a crucial time period around the beginning of light exposure. One of the most important issues surrounding the findings from the

work done on N/OFQ is illustrated in Figure 14A, but is also highly relevant for the intra-SCN GABAergic synapses displayed in Figs. 14 B, C, namely the projection responsible for the release of N/OFQ in the SCN. Fig. 14A shows a representative putative projection—discussed below—from the intergeniculate leaflet (IGL) that might be responsible for the activity of N/OFQ at either pre- or postsynaptic receptors. Another possible scenario would have it that N/OFQ is actually co-released from vesicles along with glutamate, similar to the peptide PACAP. However, while PACAP facilitates glutamatergic neurotransmission by enhancing the current through postsynaptic AMPA receptors (Kopp et al., 2001), N/OFQ inhibits glutamatergic neurotransmission at this synapse by inhibiting presynaptic N and P/Q type voltage-activated Ca^{2+} channels (Manuscript #3) and evoking a postsynaptic, hyperpolarization K^+ current (Allen et al., 1999).

Figure 14B illustrates the issues surrounding the observed short-term synaptic plasticity (Manuscript #1). Here, a presynaptic cell is firing action potentials at an avaerage rate of daytime action potential firing (trace adapted from Kononenko et al., 2004) that presumably leads to the release of GABA at that frequency. The interlocked half-circle next to the GABA-containing vesicles indicates that GABA content in the SCN varies over the diurnal cycle (see below: Aguilar-Roblero et al., 1993; Cagampang et al., 1996; Huhman et al., 1999), and this might be one reason for the observation of type 1 PPD in the SCN (Manuscript #1). Figure 14B also illustrates that the presynaptic vesicle docking and release proteins SNAP25 and Munc18 exhibit a circadian rhythm in expression (Panda et al., 2002a), which might be responsible for the diurnal rhythm of the observed type 2 PPD in the SCN (Manuscript #1). Finally, Figure 14B depicts the possibility that GABA might act as an excitatory

neurotransmitter at certain times of the circadian cycle (Wagner et al., 1996; de Jeu and Pennartz 2002) by schematically indicating the putative mechanism both of these groups proposed: the modulation of inward and outward Cl^- pumps over the course of the circadian cycle. This modulation—represented by the interlocked half-circles in the schematic—is proposed to change the reversal potential for Cl^- in SCN neurons such that activation of the Cl^- specific $GABA_A$ channel would lead to a depolarization when Cl^- travels out of the pore and a hyperpolarization when Cl^- travels into the pore. This, possibly coupled with a slight change in membrane voltage (Jiang et al., 1997) and the possibility that the $GABA_A$ mediated current is partially carried by bicarbonate (Wagner Sagiv and Yarom, 2001) could lead to the excitatory effects of GABA in the SCN.

Figure 14C depicts the same intra-SCN synapse, as Fig. 14B, but schematically represents the issues involved in the retrograde inhibition portion of this work. A number of these issues will be discussed below, including the question about the identity of the retrograde messenger and the presynaptic G-protein coupled receptor mechanism, which is related to the search for the cell type that employs retrograde inhibition. One possible confound of the results presented is indicated in this figure. That is, based on the observation that $GABA_A$ receptors associate with clathrin (Tehrani and Barnes, 1993), and that clathrin-mediated endocytosis is highly regulated by intracellular Ca^{2+} from voltage-activated channels (Cousin, 2000), it is possible that the effect of depolarization-induced suppression of the GABAergic current in the SCN might be mediated by a postsynaptic endocytosis of $GABA_A$ channels. The experiments proposed below to identify the retrograde neurotransmitter responsible for depolarization-induced inhibition might help to partially clarify this issue.

Short-term plasticity:

The results in this section were obtained from animals kept in a 12-hour light / 12-hour dark cycle, and it is therefore unclear whether the observed short-term synaptic plasticity varies with circadian time or as a result of the SCN being a retinorecipient area. To illustrate the distinction, it is valuable to examine another finding that was thought at first to be due to the circadian clock but was later shown to be present only in animals kept in a diurnal light/dark cycle. The original data showed that a day/night variation existed in GABA content in the SCN—both in terms of GAD (Cagampang et al., 1996) and GABA (Aguilar-Roblero et al., 1993)—and the interpretation was that this finding was due to the circadian clock. However, it was later shown that the GABA rhythm is blocked in the absence of retinal light inputs (Huhman et al., 1999). It would be important to determine if the diurnal variation in the observed short-term synaptic plasticity is also present in animals kept in constant darkness to answer the question of whether the effect can be linked to the circadian clock. Short-term synaptic plasticity has been implicated in balancing inhibitory and excitatory neurotransmission (Galarreta and Hestrin, 1998), and in gain control of synaptic transmission so that postsynaptic cells are desensitized to synaptic inputs at regularly recurring frequencies but are more sensitive to changes in these frequencies as a result (Abbott et al., 1997). Either or both of these could conceivably be the function of short-term plasticity in the SCN, and finding whether the circadian clock or whether retinal light inputs control the expression of this would allow us to hypothesize a role for short-term synaptic plasticity in the SCN circuit. For instance, synaptic plasticity due to regular light inputs could be viewed as the mechanism by which a shift in the balance between inhibitory and excitatory inputs is made possible at the GABAergic synapse. Alternatively, the circadian clock

might provide gain control in synaptic GABAergic neurotransmission by controlling both the rise and fall of action potential firing frequencies and the expression of synaptic plasticity. This could give valuable information about how synaptic transmission influences intra-SCN cellular coupling.

Depolarization-induced inhibition

From the presented data, it is clear that depolarization-induced Ca^{2+} influx mediates depolarization-induced inhibition in the SCN. However, it has not yet been shown whether there is a threshold in the number of action potentials required to elicit depolarization-induced inhibition. To examine this issue, the depolarization protocol should be changed to resemble the action potential waveform rather than the square depolarization protocol used in the present study. Similar to determining the number of action potentials, one could also determine a threshold action potential firing frequency required to elicit depolarization-induced inhibition. Determining a threshold action potential number and frequency could provide information as to the circumstances under which depolarization-induced inhibition can occur in the SCN. An extension to this series of experiments would be to examine a threshold Ca^{2+} level in the dendrite that is required to elicit depolarization-induced inhibition. These experiments could be performed by simultaneously recording the electrophysiological currents and intracellular Ca^{2+} levels with an indicator dye present in the internal solution.

Finally, identification of the retrograde messenger and the cell types involved in depolarization-induced inhibition is a critical element in any future study of this phenomenon in the SCN. These studies could be performed using specific candidate antagonists and confirming these results with dual labeling of the recorded cell (labeling

the recorded cell with, for example, neurobiotin and probing for the candidate neurotransmitter). The neurobiotin loading could also allow insight into the types of cells involved. If a clear cell type can be identified, hypotheses could be developed as to the effect of SCN microinjection of the retrograde transmitter's antagonist on *in vivo* circadian rhythms.

N/OFQ

The afferent projection to the SCN responsible for release of N/OFQ remains a mystery. Answering this question could help explain under what physiological conditions the peptide would be expected to act *in vivo*. Though there are other possible sources for the N/OFQ, the most reasonable is a projection from a remote brain area. A less attractive model would have SCN neurons themselves produce N/OFQ, since no evidence has been found for precursor peptide mRNA expression in the SCN (Allen et al., 1999). Therefore, N/OFQ signaling by SCN neurons is at best highly restricted, so that the normally low or absent mRNA and peptide expression is stimulated in very limited situations. Yet this is somewhat unlikely since control over peptide production and release traditionally usually lies at the post-translational stage (de Wied, 1987). Another possibility would be that a neighboring hypothalamic region such as the paraventricular nucleus might release N/OFQ into the third ventricle to act in a hormone-like fashion, and that this release could activate receptors in the SCN (Darland et al., 1998). However, its role in modulating synaptic transmission suggests a more specific role—possibly as a co-factor released with another neurotransmitter—rather than a generalized hormone-like role that is not specific to the SCN.

Assuming that N/OFQ is likely to be released from afferents from other brain regions, there are several candidate regions that could be responsible. Retrograde labeling experiments have shown that the SCN receives neural inputs from various areas in the central nervous system. These areas include the infralimbic cortex, lateral septum, medial preoptic area, subfornical organ, paraventricular thalamus, subparaventricular zone, ventromedial hypothalamic nucleus, posterior hypothalamic area, intergeniculate leaflet, olivary pretectal nucleus, ventral subiculum, and the median raphe nuclei (Moga and Moore, 1997). Some N/OFQ expression patterns correlate with the projection patterns of SCN inputs such as the limbic system, the hypothalamus, and visual afferents (Boom et al., 1999).

Of the prominent projections into the SCN, a very likely candidate for release of prepro OFQ-derived peptides is the projection from the intergeniculate leaflet. First, a high level of the precursor prepro N/OFQ mRNA was found in the ventrolateral geniculate nucleus (Boom et al., 1999), a region that is often indistinguishable from the IGL. Secondly, certain IGL projection neurons likely contain an additional neurotransmitter—possibly a peptide—besides VIP and GABA (Moore and Card, 1994). Another candidate projection area is the raphe that is responsible for the 5-HT projection into the SCN (Leander, Vrang and Moller, 1998), though most neurons expressing the prepro-OFQ mRNA in this region are small, interneuron-like neurons that are unlikely candidates for projection to other areas (Ikeda et al., 1998). The projection from the lateral septum might also be considered as a candidate, since cells labeled with prepro OFQ mRNA there are large, projection-type neurons (Ikeda et al., 1998) and a high to moderate expression level was found in this region (Boom et al., 1999).

The locus (or loci) of N/OFQ production relevant to circadian physiology will lead to significant insight into the physiological role of this peptide in circadian function. One global role for the N/OFQ peptide has been shown to be in mediating an organism's stress response (Jenck et al., 1997; Gavioli et al., 2002), though the recruitment of N/OFQ may not be triggered under all stressful situations (Ouagazzal et al., 2003). In the studies mentioned above, it is generally observed that the lack of an intact N/OFQ system (meaning the peptide and/or receptor) in the central nervous system leads to an increased stress-related response. As far as the SCN is concerned, it is known that the SCN can exert control over the day-night rhythm of corticosterone release (Inouye and Shibata, 1994) and that this is the result of a synaptic circuit to the HPA axis (Buijs et al., 1999). Hence, the SCN may exert some influence on the response to stressful stimuli. Alternatively, the stress response itself shapes the ability of the SCN to respond to light. Specifically, light-induced phase delays are inhibited in animals presented with novel environmental stressors (Amir and Stewart, 1998). Given the similarity of the effect of N/OFQ microinjected into the SCN (Allen et al., 1999), this may be mediated by the inhibition of synaptic transmission by N/OFQ in the SCN.

In conclusion, my thesis research has advanced the knowledge of synaptic communication in the SCN by displaying three modulatory mechanisms at SCN synapses. These mechanisms affect the strength of synaptic inputs onto SCN neurons and thereby could affect information processing relevant to signal sensitivity and action potential firing patterns. The role of the peptide N/OFQ in the SCN has also been expanded to show its influence on synaptic transmission. Each of these mechanisms of modulation at SCN synapses demonstrate how the effects of the relatively short-

duration of individual synaptic events can transmit useable information that allows the integration of the longer-term 24 hour rhythms. Although the SCN employs a wide variety of mechanisms to communicate rhythmic information—including diffusible factors and gap junctions briefly described above—synaptic transmission in the SCN is both subject to the influence of the circadian clock and helps form its output. Therefore, modulatory mechanisms such as changes in synaptic strength over the course of the circadian cycle, direct retrograde inhibition of synaptic inputs, and the effects of peptides such as N/OFQ likely play an integral role in providing the longer-term relevance of these shorter- term signaling events.

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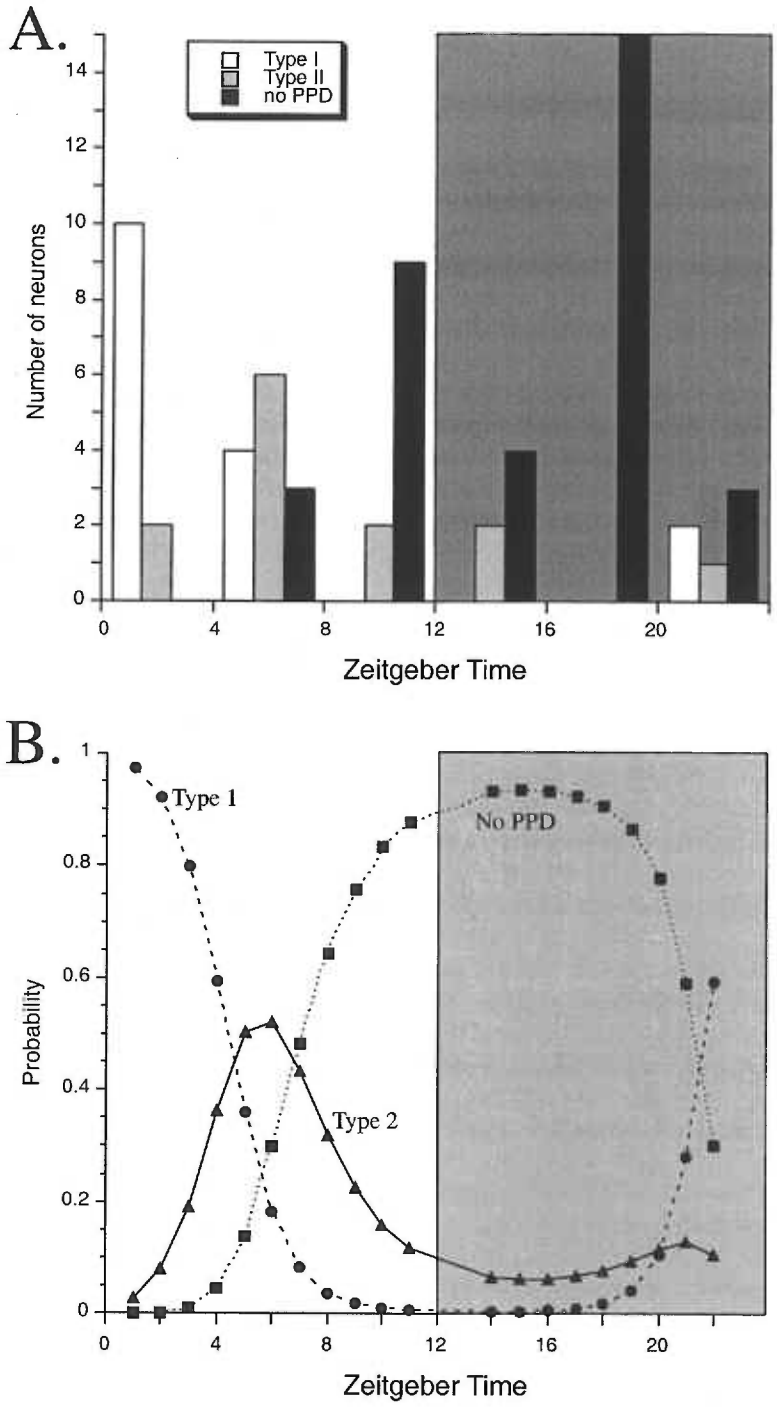
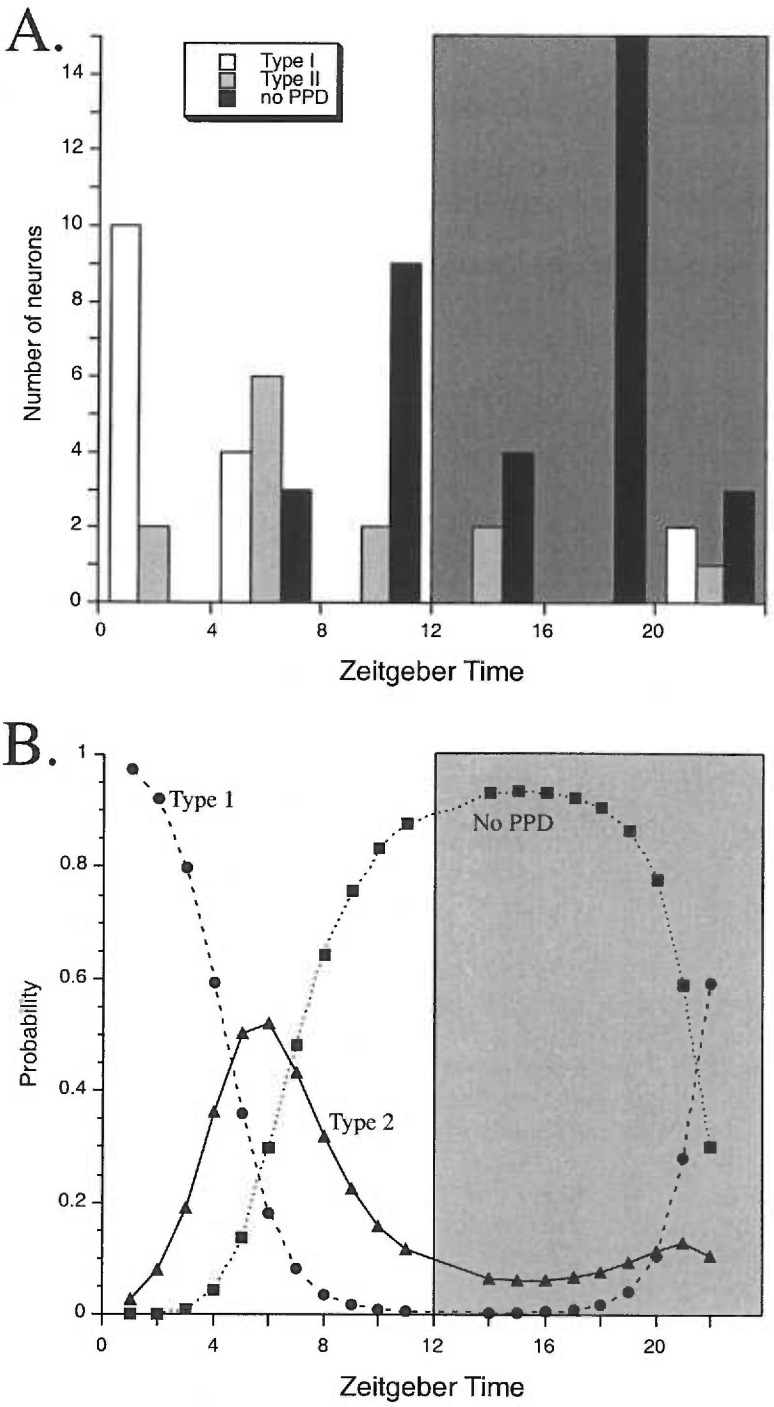


Figure 2. Diurnal rhythm of PPD in the SCN. A. Total number of neurons of Type 1 (open bars, n = 16), Type 2, (shaded bars, n = 13), and no PPD (black bars, n = 34) for each four hour bin of the 24 hour day. B. Plot of the log-linear model fitting the number of synapses exhibiting Type 1 (circles), Type 2 (triangles), or no PPD (squares) for each one-hour bin. Over 24 hours, the probability that any given synapse exhibits a particular response type strongly varies, ($X^2_{34} = 27.25$, $p = 0.787$; Deviance goodness-of-fit test) such that Type 1 responses are most likely found during the late night (ZT 20) to early morning (ZT 4), Type 2 during the mid-day (ZT 2-10) and no PPD responses are found primarily at night.



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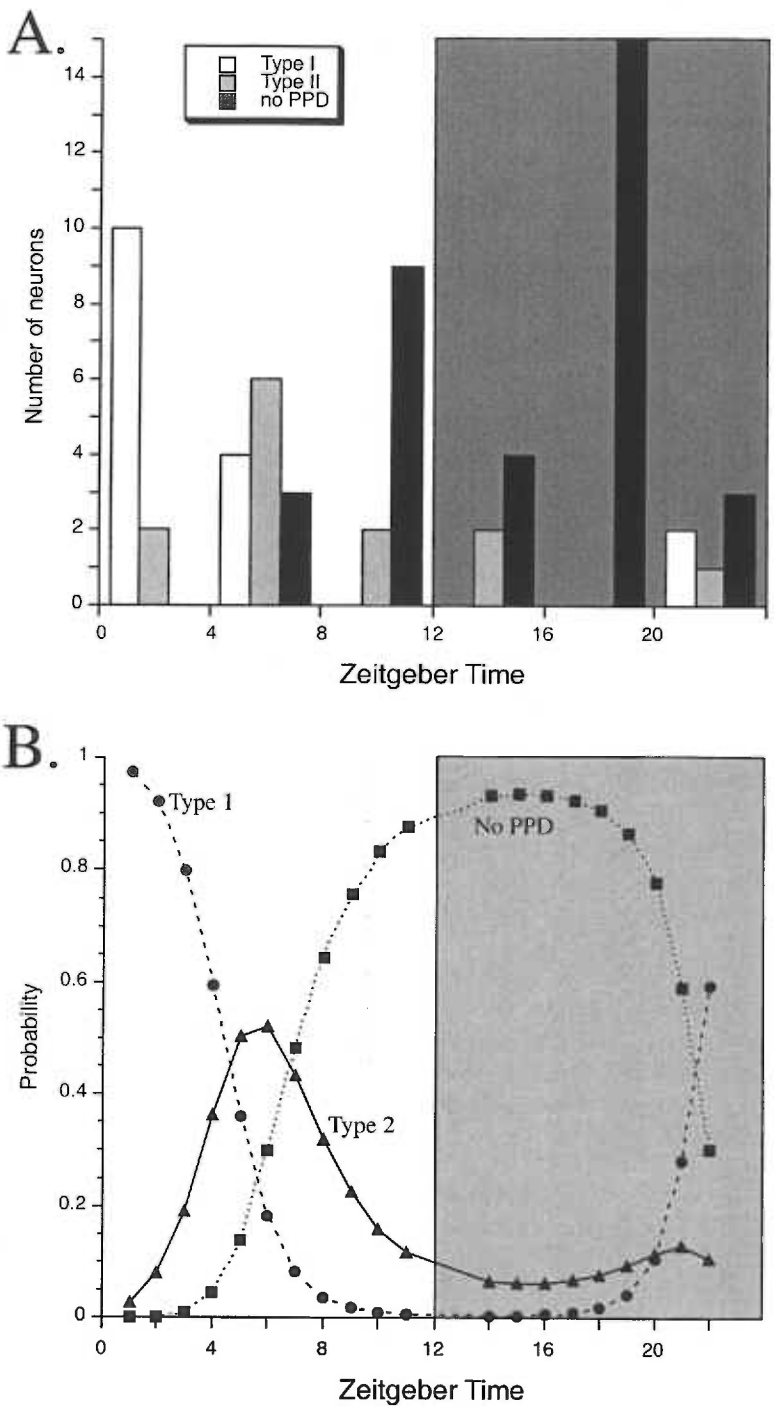

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