Glutamate Blocks Serotonergic Phase Advances of the Mammalian Circadian Pacemaker through AMPA and NMDA Receptors

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The phase of the mammalian circadian pacemaker, located in the suprachiasmatic nucleus (SCN), is modulated by a variety of stimuli, most notably the environmental light cycle. Light information is perceived by the circadian pacemaker through glutamate that is released from retinal ganglion cell terminals in the SCN. Other prominent modulatory inputs to the SCN include a serotonergic projection from the raphe nuclei and a neuropeptide Y (NPY) input from the intergeniculate leaflet. Light and glutamate phase-shift the SCN pacemaker at night, whereas serotonin (5-HT) and NPY primarily phase-shift the pacemaker during the day. In addition to directly phase-shifting the circadian pacemaker, SCN inputs have been shown to modulate the actions of one another. For example, 5-HT can inhibit the phase-shifting effects of light or glutamate applied to the SCN at night, and NPY and glutamate inhibit phase shifts of

one another. In this study, we explored the possibility that glutamate can modulate serotonergic phase shifts during the day. For these experiments, we applied various combinations of 5-HT agonists, glutamate agonists, and electrical stimulation of the optic chiasm to SCN brain slices to determine the effect of these treatments on the rhythm of spontaneous neuronal activity generated by the SCN circadian pacemaker. We found that glutamate agonists and optic chiasm stimulation inhibit serotonergic phase advances and that this inhibition involves both AMPA and NMDA receptors. This inhibition by glutamate may be indirect, because it is blocked by both tetrodotoxin and the GABA_A antagonist, bicuculline.

Key words: *suprachiasmatic; circadian; serotonin; glutamate; NMDA; AMPA; tetrodotoxin; DPAT; bicuculline; brain slice; rat*

The suprachiasmatic nucleus (SCN) contains the primary circadian clock in mammals (Moore, 1995). The SCN pacemaker generates sustained near-24 hr oscillations *in vitro* when maintained in culture in the absence of synchronizing stimuli (Shinohara et al., 1995; Yamazaki et al., 2000). Under normal conditions, however, SCN pacemaker phase is modulated by a variety of signals. These signals are generally divided into two categories: photic signals that modulate circadian phase when presented during the subjective night and nonphotic signals that modulate circadian phase when presented during the subjective day. The best characterized photic signal is light itself, which induces phase delays during the early night and phase advances during the late night (Takahashi and Zatz, 1982). These effects are mimicked by injecting glutamate or its agonists into the SCN (Mintz et al., 1999) or applied *in vitro* (Ding et al., 1994, 1997). Light and glutamate are thought to modulate the clock through activating NMDA and non-NMDA glutamate receptors, increasing intracellular Ca²⁺, and activating nitric oxide synthase (Ding et al., 1994, 1997, 1998; Hamada et al., 1999; Mintz et al., 1999). Glutamate and light also activate cAMP response element-binding protein, increase c-fos levels, and increase levels of the circadian clock-associated gene products mPER1 and mPER2, any or all of which may be critical for photic phase shifts (Ding et al., 1997; Akiyama et al., 1999; Francois-Bellan et al., 1999; Obrietan et al., 1999; Field et al., 2000).

Nonphotic stimuli, conversely, phase-advance the SCN pace-

maker when applied during the subjective day and generally have smaller effects at night. These stimuli include behavioral activity (Mrosovsky, 1995), sleep deprivation (Antle and Mistlberger, 2000; Grossman et al., 2000), and application of neuropeptide Y (NPY) (Biello et al., 1994; Golombek et al., 1996), melatonin (Cassone et al., 1985; Gillette and McArthur, 1996), GABA (Smith et al., 1989; Biggs and Prosser, 1998), or serotonin (5-HT) agonists (Medanic and Gillette, 1992; Shibata et al., 1992b; Tominaga et al., 1992; Edgar et al., 1993; Prosser et al., 1993) to the SCN.

Recent investigations have demonstrated interactions between phase-shifting stimuli. Most notably, several nonphotic stimuli (e.g., wheel-running behavior, 5-HT agonists, and NPY) have been shown to inhibit light- and/or glutamate-induced phase shifts (Ralph and Mrosovsky, 1992; Pickard et al., 1996; Biello et al., 1997; Pickard and Rea, 1997a; Mistlberger and Antle, 1998; Weber et al., 1998; Yannielli and Harrington, 2000). Glutamate and light, in turn, can block NPY-induced phase shifts (Biello et al., 1997), and light inhibits activity-arousal-induced phase shifts (Mrosovsky, 1991; Biello and Mrosovsky, 1995; Antle and Mistlberger, 2000; Grossman et al., 2000). To further explore photic-nonphotic interactions, the experiments presented here focus on whether glutamate can inhibit serotonergic phase shifts *in vitro*. The results indicate that glutamate inhibits serotonergic phase advances through stimulating both AMPA and NMDA receptors, and this effect is mimicked by electrical stimulation of the optic chiasm. In addition, the inhibition by glutamate appears to be indirect, possibly involving GABA interneurons.

MATERIALS AND METHODS

Brain slice preparation. Coronal brain slices (500 μ m) containing the SCN were prepared during the daytime from adult, male Sprague Dawley rats housed in a 12 hr light/dark cycle as reported previously (Prosser and

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Gillette, 1989; Prosser et al., 1993; Prosser, 1998b). Slices were maintained at the interface of a Hatton-style brain slice chamber (Hatton et al., 1980) in which they were perfused continuously with warm (37°C), oxygenated (95% O₂/5% CO₂), glucose–bicarbonate-supplemented Earle's Balanced Salt Solution (EBSS; Sigma, St. Louis, MO), pH 7.4–7.5.

Single-unit recordings and data analysis. Single-unit recordings were obtained using methods described previously (Prosser et al., 1993; Prosser, 1998b). Briefly, the spontaneous activity of single SCN neurons was recorded using glass capillary microelectrodes filled with 3M NaCl. Each neuron was recorded for 5 min, and the data was stored for later determination of firing rate using a DataWave system (DataWave Technologies, Longmont, CO). In general, four to seven cells were recorded during each hour. These firing rates were used then to calculate 2 hr running averages, lagged by 1 hr, to obtain a measure of population neuronal activity. As in previous studies (Prosser et al., 1993; Prosser, 1998b), the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity.

Experimental treatments. All drugs used in phase-shifting experiments were bath-applied during the first day *in vitro* by stopping the perfusion and replacing the medium in the slice chamber with medium containing the test compound. At the end of the treatment period, the normal medium was reintroduced into the slice chamber, and perfusion was resumed. In most experiments, the treatment period lasted 1 hr, but in some experiments a 10 min application was used. In another group of experiments, the medium in the slice chamber was replaced three times during the hour-long treatment. This method was initially used to test the blocking effects of glutamate after the 1 hr bath application was found ineffective (see Table 1). The reasoning behind this was that the glutamate might undergo rapid degradation and/or sequestration during the static bath conditions (Yudkoff et al., 1994; Hertz et al., 1999). This method of drug application produced positive results with glutamate, but not NMDA or kainate (see Table 1). For blocking experiments, the bathing medium was first replaced with medium containing the blocking compound. After 15 min (or after 5 min, if using the shorter, 10 min treatment paradigm), this solution was replaced with medium containing both compounds. This was followed by another 15 min (or 5 min) treatment with medium containing only the blocking agent, after which the normal medium was reintroduced to the slice chamber, and perfusion was resumed. These procedures have been shown not to induce phase shifts by themselves. Therefore, the times-of-peak for drug-treated slices were compared with the mean time-of-peak for untreated slices [zeitgeber time (ZT) 6.0 ± 0.3, *n* = 3, where ZT0 is the time of lights-on in the animal colony] to determine the amount of phase shift induced by the treatment. ANOVAs and Student's *t* tests were used, where appropriate, to test for significant differences between the means, with significance set at *p* < 0.05. Chemicals used in the study were (+)-8-hydroxy-dipropylaminotetralin HBr [(+)-DPAT], tetrodotoxin (TTX), 5-hydroxytryptamine (5-HT), bicuculline methiodide, L-glutamate, NMDA, AMPA, and kainate (Research Biochemicals, Natick, MA; Sigma).

Optic chiasm stimulation. Optic chiasm stimulation (OCS) was performed as described previously (Prosser, 1998a). Briefly, a bipolar, blunt-cut, insulated tungsten electrode was positioned in the optic chiasm ventrolateral to the SCN. Voltage (10 Hz, 10V, 3 msec duration) was applied for 15 min to determine the effect of stimulation alone. When combined with drug application, stimulation was applied first for 5 min, then in combination with the drug for 10 min, followed by a 5 min period of stimulation alone.

Multiunit activity recordings. Multiunit activity (MUA) recordings were used to determine the acute effects of experimental compounds on SCN neuronal activity. For these, a single 76 μm diameter blunt-cut, Teflon-coated metal electrode (90% platinum/10% iridium) was used as described previously (Prosser, 1998a). The electrode was first placed in the optic chiasm to determine the level of background electrical noise, and then it was moved to the SCN and lowered 50–100 μm into the slice. A threshold for counting electrical events (neuronal activity) was set to at least twice the level of background noise. Neuronal activity, expressed as the number of threshold crossings per second, was monitored continuously using a DataWave data collection and analysis system. After the MUA recording stabilized, drugs were rapidly perfused into the brain slice chamber (40 ml/hr; chamber volume, 3 ml) so that a complete exchange of the medium within the slice chamber occurred within 5 min. For each compound tested, the drug was applied for 15–30 min, followed by at least 30 min of perfusion with the normal medium. All drugs were

applied during the first day *in vitro*, between ZT4 and ZT11. Although we have found MUA recordings provide a consistent and reliable record of acute changes in neuronal activity, we are unable to obtain reliable long-term (24–48 hr) recordings using MUA (Prosser, 1998a) and therefore do not use this technique for phase-shifting experiments.

RESULTS

5-HTergic phase advances at ZT6

Consistent with previous reports (Medanic and Gillette, 1992; Shibata et al., 1992b; Prosser et al., 1993), 5-HT (10 μM) and (+)-DPAT (10 μM) induced robust phase advances when bath-applied to SCN slices for 1 hr at ZT6 (Fig. 1). Similar phase

Figure 1. Serotonergic phase advances of the SCN neuronal activity rhythm. Shown are the 2 hr means ± SEM of SCN neuronal activity obtained in a control experiment (A), after treatment with 10 μM 5-HT (B), and after treatment with (+)-DPAT (10 μM) (C). Neuronal activity peaked near ZT6 in the control experiment, whereas the peak in neuronal activity occurred at ZT2 after both the 5-HT and (+)-DPAT treatment. Thus, both 5-HT and (+)-DPAT phase-advanced the neuronal activity rhythm by 4 hr. Horizontal bars, Time of lights-off in the animal colony; vertical bar, time of drug treatment; dotted line, mean time-of-peak in control experiments.

Table 1. Summary of the phase-shifting effects of various compounds applied *in vitro*

Treatment	Number	Length of treatment	Phase shift ^a
5-HT (10 μ M)	3	1 hr	4.3 $\pm$ 0.2*
(+)DPAT (10 μ M) ^b	3	1 hr	3.7 $\pm$ 0.5*
(+)DPAT (10 μ M)	2	10 min	4.0 $\pm$ 0.0*
Glutamate (10 mM)	2	1 hr	0.0 $\pm$ 0.0
AMPA (10 μ M)	2	1 hr	0.8 $\pm$ 0.4
NMDA (100 μ M)	2	1 hr	0.8 $\pm$ 0.4
Kainate (100 μ M)	2	1 hr	0.5 $\pm$ 0.0
Optic chiasm stimulation	3	15 min	−0.2 $\pm$ 0.2
(+)DPAT (10 μ M) + glutamate (10 μ M–1 mM)	2	1 hr	2.8 $\pm$ 0.4*
(+)DPAT (10 μ M) + glutamate (10 mM)	3	1 hr	2.2 $\pm$ 0.2*
[(+)DPAT (10 μ M) + glutamate (10 mM)] $\times$ 3 ^c	2	1 hr	0.3 $\pm$ 0.4
[(+)DPAT (10 μ M) + glutamate (10 mM) + TTX (1 μ M)] $\times$ 3 ^c	3	1 hr	3.2 $\pm$ 0.1*
5-HT (10 μ M) + AMPA (10 μ M)	3	1 hr	0.3 $\pm$ 0.2
(+)DPAT (10 μ M) + AMPA (10 μ M)	3	1 hr	0.3 $\pm$ 0.2
(+)DPAT (10 μ M) + AMPA (10 μ M) + TTX (1 μ M)	3	1 hr	3.0 $\pm$ 0.4*
(+)DPAT (10 μ M) + NMDA (10–100 mM)	4	1 hr	3.1 $\pm$ 0.3*
(+)DPAT (10 μ M) + NMDA (100 μ M) + AMPA (0.1 μ M)	2	1 hr	3.1 $\pm$ 0.2*
[(+)DPAT (10 μ M) + NMDA (10 μ M)] $\times$ 3 ^c	2	1 hr	4.0 $\pm$ 0.7*
(+)DPAT (10 μ M) + NMDA (100 μ M)	3	10 min	0.1 $\pm$ 0.1
(+)DPAT (10 μ M) + NMDA (100 μ M) + TTX (1 μ M)	3	10 min	3.3 $\pm$ 0.5*
(+)DPAT (10 μ M) + bicuculline (30 μ M)	3	10 min	3.9 $\pm$ 0.1*
(+)DPAT (10 μ M) + NMDA (100 μ M) + bicuculline (30 μ M)	3	10 min	3.8 $\pm$ 0.1*
(+)DPAT (10 μ M) + kainate (10–100 μ M)	4	1 hr	3.4 $\pm$ 0.3*
[(+)DPAT (10 μ M) + kainate (100 μ M)] $\times$ 3 ^c	2	1 hr	3.3 $\pm$ 0.4*
(+)DPAT (10 μ M) + kainate (100 μ M)	2	10 min	3.5 $\pm$ 0.0
(+)DPAT (10 μ M) + optic chiasm stimulation	3	10 min	0.8 $\pm$ 0.2
(+)DPAT (10 μ M) + optic chiasm stimulation + bicuculline	3	10 min	3.17 $\pm$ 0.2*

^aPhase shifts calculated by comparing the times-of-peak in drug-treated versus control slices with the mean time-of-peak in control slices of ZT 6.0 $\pm$ 0.3 (n = 3).

^bData from Prosser (1998b).

^c" $\times$ 3" refers to three applications during the 1 hr treatment period. For details, see Materials and Methods.

* p < 0.05 versus control.

advances were also seen after 10 min bath-applications of (+)DPAT (Table 1). Conversely, neither glutamate nor any of its agonists (AMPA, NMDA, and kainate) altered the phase of the neuronal activity rhythm when applied at ZT6 (Table 1). All glutamatergic compounds were tested at concentrations that were either known to affect SCN activity during the day (Shibata et al., 1992a; Flett and Colwell, 1999) or shown previously to induce phase shifts during the night (Ding et al., 1994; Shibata et al., 1994; Forrest and Prosser, 2000) (our unpublished data). These data are summarized in Table 1.

Glutamatergic inhibition of 5-HTergic phase shifts

Although it did not induce phase shifts when applied alone at ZT6, AMPA (10 μ M) completely abolished the phase advances induced by both 5-HT and (+)DPAT under 1 hr static bath conditions (Fig. 2). AMPA inhibition of (+)DPAT-induced phase shifts was dose-dependent, with an ED₅₀ $\sim$ 1 μ M (Fig. 3). Glutamate, when reapplied three times during the course of the 1 hr treatment period (see Materials and Methods), also completely blocked (+)DPAT-induced phase advances. As with AMPA, this inhibition was dose-dependent, although much higher concentrations of glutamate were needed to block (+)DPAT-induced phase shifts (Fig. 3).

NMDA and kainate, at concentrations up to 100 μ M, were completely ineffective at blocking phase advances induced by 1 hr (+)DPAT bath application at ZT6 (Table 1). These results were surprising because NMDA and kainate receptors are abundant in the SCN (van den Pol et al., 1994, 1996; Mikkelsen et al., 1993); NMDA and kainate increase SCN neuronal activity and intracellular Ca²⁺ levels (Shibata et al., 1992a; Dudek et al., 1993; Haak, 1999); and NMDA phase-shifts the SCN pacemaker when applied during the night (Ebling et al., 1991; Ding et al., 1994, 1997; Mintz and Albers, 1997; Mintz et al., 1999; Forrest and Prosser, 2000). However, in some systems, functional activation of NMDA receptors requires concurrent AMPA receptor-induced depolarization (van den Pol et al., 1996; Dingledine et al., 1999). Therefore, we tested the ability of a combined application of AMPA (0.1 μ M) with 100 μ M NMDA. This combination still did not block (+)DPAT-induced phase advances (Table 1).

Further investigation into the effects of AMPA and NMDA on SCN neuronal activity revealed that the excitation induced by NMDA dampened rapidly, so that activity often returned to near baseline levels within 15 min of its initial application. In contrast, the excitatory response to AMPA generally lasted much longer (Fig. 4). Thus, we speculated that NMDA might be more effective

Figure 2. AMPA blocks serotonergic phase advances of the SCN neuronal activity rhythm. *A*, Coapplication of AMPA (10 μ M) blocks the 5-HT-induced phase advance. *B*, Coapplication of AMPA (10 μ M) completely abolished the (+)DPAT-induced phase advance. *C*, TTX (1 μ M) prevents the inhibition by AMPA, thus restoring the (+)DPAT-induced phase advance. See Figure 1 legend for details.

when coapplied with (+)DPAT for a shorter length of time. In fact, NMDA completely blocked the phase advances induced by 10 min bath application of (+)DPAT (NMDA applied alone for 5 min before and after (+)DPAT–NMDA treatment). Similar inhibition still was not seen when kainate was coapplied with (+)DPAT for 10 min. (Table 1).

Optic chiasm stimulation inhibits serotonergic phase shifts

Next, we investigated whether stimulation of endogenous glutamate release could block serotonergic phase advances. To test this, we applied electrical stimulation to the optic chiasm, which should stimulate release of glutamate from retinal terminals in the SCN (Liou et al., 1986). This treatment had no effect on the rhythm of SCN neuronal activity when applied alone at ZT6, but it inhibited phase advances induced by 10 min bath application of (+)DPAT (Fig. 5, Table 1).

Inhibition of DPAT-Induced Phase Shifts

Figure 3. Dose dependence of glutamatergic inhibition. Shown are the mean phase advances ($\pm$ SEM) induced by (+)DPAT application alone and in the presence of varying concentrations of AMPA (filled circles) and glutamate (open circles). Complete inhibition occurred with 10 μ M AMPA and 10 mM glutamate (when glutamate is reapplied 3 times during the 1 hr treatment period). The ED_{50} for AMPA is near 1 μ M and for glutamate is near 5 mM.

Figure 4. Multiunit activity recordings from the SCN showing acute effects of NMDA and AMPA on SCN neuronal activity. Perfusion application of NMDA to the SCN slice induced a large increase in activity that rapidly returned to near baseline levels. Conversely, neuronal activity remained high throughout the period of AMPA application.

Glutamatergic inhibition is blocked by TTX and bicuculline

Finally, we investigated whether the glutamatergic inhibition involves direct or indirect interactions with 5-HT, that is, whether the 5-HT and glutamate agonists act on the same cells. To initially address this question, we applied TTX in conjunction with glutamate, AMPA, or NMDA under the experimental conditions in

Figure 5. Optic chiasm stimulation inhibits serotonergic phase advances. *A*, Electrical stimulation of the optic chiasm at ZT6 for 15 min did not shift the rhythm of SCN neuronal activity. *B*, Optic chiasm stimulation inhibited the phase advance induced by 10 min application of (+)DPAT, so the peak in neuronal activity occurred near ZT6. *C*, Bicuculline coapplied with OCS reinstates the (+)DPAT-induced phase advance. See Figure 1 legend for details.

which each compound had been found to block (+)DPAT-induced phase advances. In all cases, TTX prevented the glutamatergic inhibition, so that the (+)DPAT-induced phase advance was reinstated (Figs. 2, 6; Table 1). Application of TTX alone at ZT6 does not induce phase shifts (Bergeron et al., 1999), and TTX does not block serotonergic phase shifts at ZT6 (Prosser et al., 1992).

These results suggest that the inhibition by glutamate and its agonists requires Na^+ -dependent action potentials to be formed. If this is the case, then the inhibition by glutamate may involve SCN interneurons. To address this possibility, we tested whether the glutamatergic inhibition was sensitive to blockade of GABA receptors. We have previously shown that the selective GABA_A antagonist, bicuculline, does not induce phase shifts when applied

Figure 6. Glutamate inhibition of (+)DPAT-induced phase advances is blocked by TTX. Shown are the mean phase advances ($\pm$ SEM) induced by (+)DPAT alone or in the presence of glutamate agonists with and without TTX present. The inhibition by glutamate, AMPA, and NMDA are all prevented when TTX is coapplied. Numbers under the bars indicate the number of experiments.

to the SCN *in vitro* at ZT6 (Bergeron et al., 1999). In these experiments we first tested whether bicuculline affects phase advances by (+)DPAT. As seen in Figure 7, bicuculline (30 μM) did not block (+)DPAT-induced phase shifts, but it did block the inhibition by NMDA (Fig. 7). To further investigate this effect, we tested whether bicuculline also prevents OCS inhibition of (+)DPAT-induced phase shifts. As shown in Figure 5, coapplication of bicuculline with OCS and (+)DPAT reinstates the full 5-HTergic phase advance. These data are summarized in Table 1.

DISCUSSION

These data are the first to reveal glutamatergic inhibition of serotonergic phase shifts *in vitro*, demonstrating that this inhibition takes place in or near the SCN. As such, they extend the body of research showing a pattern of inhibitory interactions between photic and nonphotic stimuli. Furthermore, the results suggest that this inhibition may also occur in response to electrical stimulation of the optic chiasm.

In our experiments, we were able to inhibit the serotonergic phase shifts through stimulation of either NMDA or AMPA receptors, but not through stimulation of kainate receptors. This is similar to the results of studies investigating photic phase shifts, in which stimulation of both NMDA and AMPA receptors can mimic light-induced phase shifts. The ability of kainate to induce photic phase shifts has not, to our knowledge, been reported. Although we cannot completely rule out involvement of kainate receptors in the inhibitory actions seen here, none of the treatment regimens we tried were effective with kainate.

Figure 7. Bicuculline (Bic) prevents glutamatergic inhibition of (+)DPAT-induced phase shifts. Shown are the mean phase advances ($\pm$ SEM) induced by (+)DPAT in the presence of NMDA and/or bicuculline. Bicuculline did not affect the (+)DPAT-induced phase advance but did prevent the inhibition by NMDA. See Figure 6 legend for details.

A large number of *in vivo* studies have shown that 5-HT can inhibit photic phase shifts. The evidence strongly supports a combination of presynaptic and postsynaptic sites of 5-HT action. Presynaptically, 5-HT stimulates 5-HT_{1B} receptors that are located on retinal ganglion cell terminals in the SCN to inhibit light-induced glutamate release (Pickard and Rea, 1997b; Pickard et al., 1999). 5-HT also appears to act postsynaptically on either 5-HT_{1A} or 5-HT₇ receptors to block the phase-shifting actions of glutamate (Rea et al., 1994, 1995; Moriya et al., 1996; Weber et al., 1998; Smith et al., 2001). The results of this study demonstrate the reverse situation, i.e., that glutamate can inhibit serotonergic phase shifts. These results are consistent with recent *in vivo* experiments showing that light can inhibit daytime phase advances induced by DPAT perfusion into the SCN (Ehlen et al., 2001). Interestingly, Challet et al. (1998) found that phase shifts induced by DPAT injection in the intergeniculate leaflet were blocked by light, whereas phase shifts induced by SCN injection of DPAT were not.

The mutual inhibition between light–glutamate and 5-HT is similar to that previously shown for light–glutamate and NPY (Biello et al., 1994, 1997; Yannielli and Harrington, 2000). Thus, it may be that in the SCN there is a general pattern of mutual inhibition between photic and nonphotic stimuli. The frequency with which interactions between phase-shifting stimuli have been observed raises interesting questions concerning how animals normally synchronize their daily rhythms to the environment. Results such as these lend support to the idea that synchronization involves a complex integration of multiple stimuli rather than

an overriding reliance on a single environmental cue such as light. The interactions observed between phase-shifting stimuli acting within the SCN are also quite interesting because they now include two examples of a neurotransmitter (glutamate) modulating the actions of neuromodulators (5-HT and NPY). Results such as these raise the general issue of whether a clear functional distinction can be drawn between classical “neurotransmitters” and “neuromodulators”.

Previous studies have demonstrated that light can inhibit activity–arousal-induced phase shifts (Mrosovsky, 1991; Biello and Mrosovsky, 1995; Antle and Mistlberger, 2000; Grossman et al., 2000). It has yet to be resolved to what extent these phase shifts occur through 5-HT, NPY, or other neurotransmitters (Biello et al., 1994; Wickland and Turek, 1994; Biello, 1995; Bobrzynska et al., 1996; Antle et al., 1998; Mistlberger and Antle, 1998). It is likely that diverse neural substrates underlie these phase shifts and that their specific roles vary somewhat between different species and between the types of arousal being investigated. The data presented here show that photic inhibition of activity-induced phase shifts in rats could involve glutamate acting within the SCN to inhibit postsynaptic actions of 5-HT. Whether or not this occurs *in vivo* and whether this underlies some aspects of photic inhibition of activity-induced phase shifts remains to be determined.

The results presented here are especially interesting in light of a recent study showing that sleep deprivation, which induces robust daytime phase advances, increases 5-HT release in the SCN by 160%, and further, that light inhibits the sleep deprivation-induced phase shifts but not the release of 5-HT in the SCN (Grossman et al., 2000). Although the inhibition by light could involve neural substrates outside the SCN, our results indicate that the inability of light to suppress 5-HT release in the SCN does not exclude the SCN from being the site of light inhibition of the phase shifts. Light could be inhibiting the phase shifts by blocking postsynaptic actions of 5-HT in the SCN. This interpretation is also consistent with data showing that light inhibits DPAT-induced phase shifts *in vivo* (Ehlen et al., 2001).

Previous work has demonstrated that serotonergic phase shifts are not blocked by TTX (Prosser et al., 1992), suggesting that 5-HT may act directly on clock cells. Likewise, *in vivo* phase shifts in response to intra-SCN injections of glutamate are not blocked by TTX, indicating that glutamate may also act directly on clock cells to phase-shift the circadian pacemaker (Mintz et al., 1999). However, the results presented here show that the inhibition of serotonergic phase shifts by glutamate is blocked by TTX. This suggests that the inhibition by glutamate requires Na⁺-dependent action potentials and is indirect. This could mean that interneurons within the SCN are required to convey the glutamate signal to 5-HT-stimulated cells. If that is the case, then it is likely that the intervening neurons use GABA as their neurotransmitter, because of its ubiquitous presence in SCN cells (van den Pol et al., 1996). Our results showing that bicuculline prevents the glutamatergic inhibition are consistent with this hypothesis. To further test this hypothesis, one would like to determine whether GABA blocks 5-HT-induced phase shifts. This experiment will be difficult, however, because we have shown that activation of both GABA_A and GABA_B receptors in the SCN induces phase advances in the subjective day at the same circadian phases that 5-HT induces phase shifts (Biggs and Prosser, 1998; Bergeron et al., 1999).

Finally, our experiments show that electrical stimulation of the optic chiasm also blocks serotonergic phase shifts. One concern

with this procedure is that the electrical stimulation may induce nonspecific release of neurotransmitters in the SCN slice. There are two reasons why we do not think that is occurring here. First, general depolarization would be expected to induce release of GABA, the most abundant neurotransmitter in the SCN (van den Pol and Dudek, 1993). However, GABA induces robust phase advances in the SCN at ZT6 (Biggs and Prosser, 1998; Bergeron et al., 1999), whereas optic chiasm stimulation does not. Second, we find that the electrical stimulation parameters used in these experiments induce phase shifts at night that mimic the phase-shifting effects of glutamate (T. Braden, V. McMillan, and R. A. Prosser, unpublished data). Together, we interpret these data as supporting the conclusion that optic chiasm stimulation blocks DPAT-induced phase shifts through inducing the release of endogenous glutamate.

In summary, this study presents evidence that glutamate, acting through both AMPA and NMDA receptors, can block the phase-modulating effects of serotonin in the SCN *in vitro*. This inhibition is prevented by coapplication of either TTX or the GABA antagonist, bicuculline, suggesting there is an indirect interaction between glutamate and serotonin with respect to phase-shifting the SCN pacemaker. These results are consistent with previous work showing mutually inhibitory interactions between photic and nonphotic stimuli in modulating the phase of the mammalian circadian pacemaker.

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