Serotonin Modulation of Calcium Transients in Cells in the Suprachiasmatic Nucleus

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Abstract Information about environmental lighting conditions is conveyed to the suprachiasmatic nucleus (SCN), at least in part, via a glutamatergic fiber pathway originating in the retina, known as the retinohypothalamic tract (RHT). Previous work indicates that serotonin (5HT) can inhibit this pathway, although the underlying mechanisms are unknown. The authors became interested in the possibility that 5HT can inhibit the glutamatergic regulation of Ca^{2+} in SCN neurons and, by this mechanism, modulate light-induced phase shifts of the circadian system. To start to examine this hypothesis, optical techniques were used to measure Ca^{2+} levels in SCN cells in a brain slice preparation. First, it was found that 5HT produced a reversible and significant inhibition of Ca^{2+} transients evoked by synaptic stimulation. Next, it was found that 5HT did not alter the magnitude or duration of Ca^{2+} transients evoked by the bath application of glutamate or N-methyl-D-aspartate acid (NMDA) in the presence of tetrodotoxin (TTX). The authors feel that the simplest explanation for these results is that 5HT can act presynaptically at the RHT/SCN synaptic connection to inhibit the release of glutamate. The demonstration that 5HT can have a dramatic modulatory action on synaptic-evoked Ca^{2+} transients measured in SCN neurons adds support to the notion that the serotonergic innervation of the SCN may function to regulate environmental input to the circadian system. In addition, it was found that the administration of higher concentrations of 5HT can increase Ca^{2+} in at least a subpopulation of SCN neurons. This effect of 5HT was concentration dependent and blocked by a broad-spectrum 5HT antagonist (metergoline). In addition, both TTX and the γ-amino-N-butyric acid (GABA) receptor blocker bicuculline inhibited the 5HT-induced Ca^{2+} transients. Therefore, the interpretation of this data is that 5HT can act within the SCN to alter GABAergic activity and, by this mechanism, cause changes in intracellular Ca^{2+}. It is also suggested that this 5HT-induced Ca^{2+} increase might play a role in 5HT-induced phase shifts of the SCN circadian oscillator.

Key words 5HT, calcium, circadian rhythms, serotonin, suprachiasmatic nucleus

In mammals, the part of the nervous system responsible for most circadian behavior can be localized to a pair of structures in the hypothalamus known as the suprachiasmatic nucleus (SCN). The circadian oscillator located in these cells generates a rhythm that repeats with a frequency of close to but not equal to 24 h. To function adaptively, these cells must be synchronized to the exact 24-h cycle of the

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physical world. The daily cycle of light and dark is the dominant cue used by organisms, including humans, to synchronize their biological clocks to the environment. Thus, a major goal of our research is to understand the cellular/molecular mechanisms by which environmental signals act to synchronize circadian oscillators.

The SCN receive light information directly through a monosynaptic projection from the retina known as the retinohypothalamic tract (RHT). The amino acid glutamate is a transmitter at the RHT/SCN synaptic connection, and this transmitter plays a critical role in mediating light regulation of the circadian system (e.g., Colwell and Menaker, 1996; Ebling, 1996). The SCN also receives a dense serotonergic projection from the midbrain raphe nuclei that terminates predominantly in the retinorecipient region of the nucleus (Moore et al., 1978). By themselves, serotonin (5HT) receptor agonists directly cause phase shifts of the circadian system both in vitro (e.g., Medanic and Gillette, 1992) and in vivo (e.g., Edgar et al., 1993). Recent evidence suggests a role for this pathway in mediating activity-induced phase shifts of the circadian system (Edgar et al., 1997; Marchant et al., 1997; Dudley et al., 1998; although see also Bobrzynska et al., 1996; Antle et al., 1998). In addition, there is evidence that this serotonergic projection can modulate light input to the SCN (Morin and Blanchard, 1991; Rea et al., 1994; Ying and Rusak, 1994; Meyer-Bernstein et al., 1997; Ying and Rusak, 1997). Thus, it appears that two major transmitter systems (glutamatergic and serotonergic) that convey information to the SCN can interact with each other, and we believe that these interactions may determine the ultimate response of the circadian system to environmental stimulation.

Among the most important consequences of glutamatergic and serotonergic regulation of neurons are the resulting changes in intracellular Ca^{2+} levels, and interactions between these transmitter systems are likely to be reflected at the level of this important signaling molecule. Light stimulation results in the release of glutamate from the retinal afferent fibers that innervate the SCN. Activation of glutamate receptors (GluRs) can cause an increase in Ca^{2+} in SCN neurons (van den Pol et al., 1992; Tominaga et al., 1994). This raises the possibility that GluR-induced increases in Ca^{2+} in the SCN are part of the mechanism that underlies light-induced phase shifts. This suggestion is supported by recent work by Gillette and coworkers, who found evidence that ryanodine receptors mediate light- and glutamate-induced phase delays of the circadian system (Ding et al., 1998). In addition, earlier work on marine mollusks indicates that a Ca^{2+} influx is required for photic regulation of the circadian system (e.g., McMahon and Block, 1987; Colwell et al., 1994). For these reasons, we felt that it was important to investigate a potential role for 5HT in modulating basal- and glutamate-stimulated Ca^{2+} levels. To address these issues, this study used optical imaging techniques and the Ca^{2+} indicator dye Fura2 to measure Ca^{2+} levels in SCN cells in a brain slice preparation.

MATERIALS AND METHODS

Animals and Brain Slice Preparation

Brain slices from rats between 10-15 days of age were prepared using standard techniques (Sprague-Dawley). For reasons that are not completely understood, infrared differential interference contrast (IR DIC) videomicroscopy and dye loading with Acetoxymethyl ester (AM) esters work better in slices from young animals. Both the circadian oscillator based in the SCN and the serotonergic innervation are known to be fully mature by this age (Reppert and Schwartz, 1984; Ugrumov et al., 1994). Rats were killed by decapitation, and brains were dissected and placed in cold oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM) NaCl 130, $NaHCO_3$ 26, KCl 3, $MgCl_2$ 5, NaH_2PO_4 1.25, $CaCl_2$ 1.0, glucose 10 (pH 7.2-7.4, osmolality 290-310 mOsm). After cutting slices, transverse sections (350 μm) were placed in ACSF (25-27°C) for at least 1 h (in this solution, $CaCl_2$ was increased to 2 mM, $MgCl_2$ was decreased to 2 mM, and 4 mM lactate was added). For specified experiments, a low-magnesium solution was used in which $MgCl_2$ was reduced to 0 mM. Slices were constantly oxygenated with 95% O_2-5% CO_2 (pH 7.2-7.4, osmolality 290-310 mOsm). Slices were placed in a custom-designed chamber attached to the stage of a fixed-stage upright microscope. The slice was held down with thin nylon threads glued to a platinum wire and submerged in continuously flowing, oxygenated ACSF (25°C) at 2 ml/min. Solution exchanges within the slice were achieved by a rapid gravity-feed delivery system. In our system, the effects of bath-applied drugs begin within 15 sec and are typically complete by 1-2 min.

IR DIC Videomicroscopy

Slices were viewed with an upright compound microscope (Zeiss Axioskop, Milpitas, CA) using a water immersion lens (40X) and DIC optics. They were illuminated with near-IR light by placing an IR band-pass filter (750-1050 nm) in the light path. The image was detected with an IR-sensitive CCD camera (Hamamatsu C2400, Bridgewater, NJ) and displayed on a video monitor. A camera controller allowed analog contrast enhancement and gain control. Digital images were stored on computer / optical disk for subsequent analysis and additional digital contrast adjustment when necessary. Cells were typically visualized from 30-100 μm below the surface of the slice.

In this study, we used IR videomicroscopy to visualize cells within the brain slice and to limit some of the uncertainty as to the cell type. This imaging technique allowed us to see clearly the SCN and to exclude cells from the surrounding hypothalamic regions. In addition, we could use morphological criteria to target SCN neurons and to avoid taking measurements from cells that were clearly glia. While size is hardly foolproof, in a few cases we have combined electrophysiological recording and fluorescent imaging to demonstrate that the cells from which we are measuring Ca^{2+} indeed show the electrical properties of neurons (n = 4). Accordingly, we believe that most of the data were collected from SCN neurons.

Calcium Imaging

A cooled CCD camera (Princeton Instruments, Microview model 1317 × 1035 pixel format) was added to the Olympus fixed-stage microscope to measure fluorescence. To load the dye into cells, slices were incubated in membrane permeable fura2-AM (50 μM) at 37°C for 10 min. The fluorescence of fura2 was excited alternatively at wavelengths of 357 nm and 380 nm by means of a high-speed wavelength-switching device (Sutter, Lambda DG-4). Image analysis software (MetaFluor, Universal Imaging) allowed the selection of several "regions of interest" within the field from which measurements are taken. To minimize bleaching, the intensity of excitation light and sampling frequency was kept as low as possible. In these experiments, the intensity of excitation light was measured as 18 μW out of the objective and measurements normally were made once every 2 sec.

Calibration of Ca^{2+} Signals

For ratiometric Ca^{2+} measurements, the dual emission dye fura2 was used. Free [Ca^{2+}] was calculated from the ratio (R) of fluorescence at 357 nm and 380 nm using the following equation: $[Ca^{2+}] = K_d \times Sf \times (R - R_{min}) / (R_{max} - R)$ (Grynkiewicz et al., 1985). Values for K_d were at 135 nM, whereas values for R_{min} and R_{max} were all determined via calibration methods. Initially, an in vitro method was used to make estimate values. With this method, rectangular glass capillaries were filled with a high Ca^{2+} (fura2 + 10 mM Ca^{2+}), a low Ca^{2+} (fura2 + 10 mM Ethylene glycol-bis(b-aminoethyl ether)-N,N,N'-tetraacetic acid [EGTA]), and a control saline without fura2. The fluorescence (F) at 380-nm excitation of the low Ca^{2+} solution was imaged, and the exposure of the camera was adjusted to maximize the signal. These camera settings were then fixed, and measurements were made with 380- and 357-nm excitation of the three solutions. $R_{min} = F357$ nm in low Ca^{2+} / F 380 in low Ca^{2+}; $R_{max} = F$ 357 in high Ca^{2+} / F 380 in high Ca^{2+}; $Sf = F$ 380 in low Ca^{2+} / F 380 in high Ca^{2+}. In addition, an in vivo calibration method was used. For this, SCN cells were loaded via the patch pipette using solutions inside the electrode similar to the normal internal solution but containing either no Ca^{2+} (20 mM EGTA) or 10 mM Ca^{2+} for R_{min} and R_{max}, respectively.

Synaptic Stimulation

Electrical stimulation was used to induce local excitatory postsynaptic synaptic currents that were mediated by GluRs. Focal stimulation was applied with a glass electrode (10 μm tip diameter) as the stimulating electrode. The electrode was placed 0.5-1.0 mm from the imaged cell and was moved around until a location was found that induced a response. Short square-wave voltage pulses (5-10 V, 200-μsec duration) were used to induce the synaptic response.

Lighting Conditions

To look for possible diurnal variation in effects of 5HT, animals were maintained on a daily 12:12 light-dark (LD) cycle. It is already well established that cells in the SCN continue to show circadian oscillations when isolated from the animal in a brain slice preparation. Accordingly, care must be taken as to the time in the daily cycle when the data are collected. Most of the animals were killed 30 min before the time that the

lights would have turned off in the LD cycle. The data from these animals were collected between zeitgeber time (ZT) 13-18 and pooled to form a "night" group. For comparison, some of the animals were killed immediately after the lights came on. The data from these animals were collected between ZT 1-6 and pooled to form a "day" group.

Statistical Analyses

Differences between experimental and control groups were evaluated using *t* tests or Mann-Whitney rank sum tests when appropriate. Values were considered significantly different if $p < .05$. All tests were performed using SigmaStat (SPSS, Chicago, IL). In the text, values are shown as mean ± SEM.

RESULTS

Data were collected from a total of 899 cells from 69 animals. All groups contained data from at least 5 animals. Each of these cells was determined to be within the SCN by directly visualizing the cell's location with IR DIC videomicroscopy before any Ca^{2+} data were collected. Small cell types including glia were easily identified and were not included in the data set. In four cases, we filled a single cell with a Ca^{2+} indicator dye by loading the dye through a patch pipette. In each case, we were able to confirm that the cells from which we were measuring Ca^{2+} could generate action potentials and thus had the electrical properties of a neuron. The stability of the Ca^{2+} measurements was determined by examining how measured values changed through time in fura2-loaded SCN cells in the absence of stimulation. Sampling every 2 sec for a total of 10 min, the baseline values changed by 4±1% after 5 min and by 8±2% after 10 min ($n = 54$). For any given cell, we defined a detectable response as one in which the change in Ca^{2+} levels was at least twice the standard deviation of our measurement of resting Ca^{2+}. On average, the resting Ca^{2+} level of these SCN cells was determined to be 90 nM with a standard deviation of 3 nM. Therefore, a typical cell had to show at least a 6 nM change from baseline in order to be judged as having a measurable response. The effects of experimental treatments were quantified by comparing the resting Ca^{2+} levels (10 sec average) in a cell before treatment to the peak Ca^{2+} level (10 sec average) after treatment, with the data typically being presented as a percentage change from baseline.

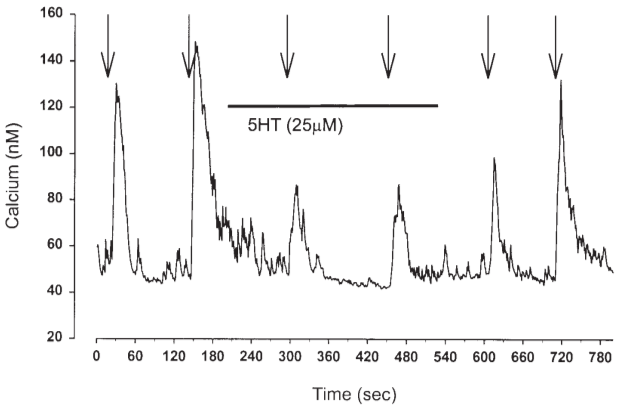

Figure 1. Synaptic stimulation–evoked Ca^{2+} transients in an SCN cell were inhibited by 5HT. Synaptic stimulation occurred at each of the arrows. Synaptic-induced increases in Ca^{2+} were partially inhibited by the NMDA antagonist AP5 (50 μM) and the α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate (AMPA/KA) GluR antagonist 6-cyano-2,3-dihydroxyl-7-nitroquinoxaline (CNQX) (data not shown). These data were collected from tissue from a 14-day-old rat in a low-magnesium solution.

5HT Inhibited Synaptic-Evoked Ca^{2+} Transients

In our first experiment, we examined the effect of 5HT on synaptic-evoked Ca^{2+} transients. These data were collected in a low-magnesium solution to unmask the contribution of NMDA receptors. Synaptic stimulation increased Ca^{2+} levels in SCN cells to 176%±22 of pretreatment controls ($n = 25$, $p < .001$; Figs. 1-2). These Ca^{2+} transients were mediated at least in part by stimulation of NMDA receptors as the bath application of the antagonist DL-2-amino-5-phosphonopentanoic acid (AP5, 50 μM, 5 min) inhibited these changes in Ca^{2+} to 61±5% of control values ($n = 8$, $p < .05$). Bath application of 5HT (25 μM, 30-60 sec) produced a reversible inhibition of the synaptic-evoked Ca^{2+} transients (Figs. 1-2). In the presence of 5HT, synaptic stimulation still increased Ca^{2+}; however, the magnitude of the response was significantly ($p < .01$) reduced from 176% to 46±11% of control levels ($n = 25$). None of the cells in this data set showed a statistically significant response to 25 μM 5HT alone (30-60 sec).

5HT Did Not Alter Glutamate-Evoked Ca^{2+} Transients

Next, we examined the effect of 5HT on glutamate and NMDA-evoked Ca^{2+} transients (Figs. 2-3). These

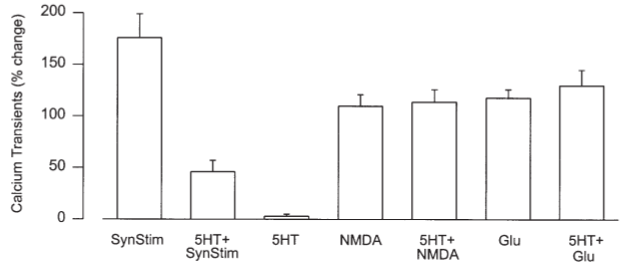

Figure 2. Histogram summarizing data examining 5HT effects on synaptic- and GluR-evoked Ca^{2+} transients in SCN cells. Ca^{2+} transients evoked by synaptic stimulation and bath application of GluR agonists were compared to those treatments in the presence of 5HT (25 μM). 5HT significantly ($p < .01$) inhibited synaptic-evoked Ca^{2+} transients but not those evoked by glutamate or NMDA. In this figure, the Ca^{2+} response is expressed as a percentage change from baseline. Error bars indicate standard error. Each group contains data from 25 to 194 cells collected from at least 5 animals (10- to 15-day-old rats).

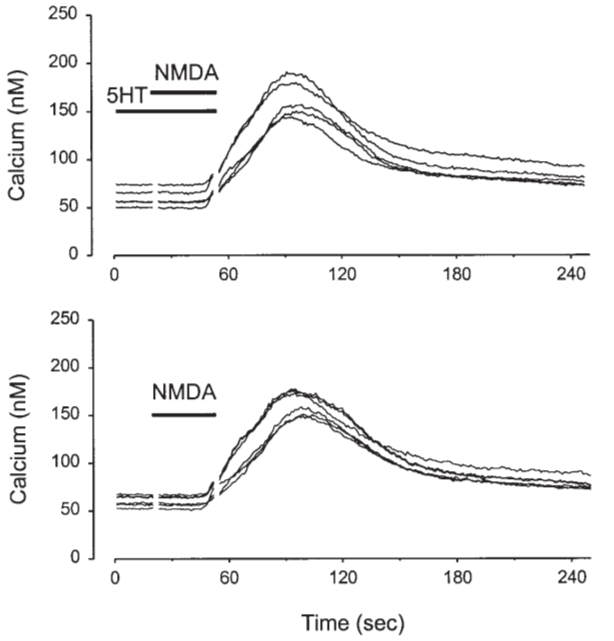

Figure 3. Example illustrating that NMDA-evoked Ca^{2+} transients in SCN cells were not inhibited by 5HT. Each line represents the Ca^{2+} levels measured in a single cell. Data from 5 adjacent cells in the SCN brain slice are shown. The cells were first exposed to the 5HT + NMDA treatment. Then, after a recovery period (3 min), these same cells were exposed to NMDA alone. There were no differences in the magnitude or duration of the Ca^{2+} transients evoked by these two treatments. Measurements of fluorescence evoked by 380- and 357-nm excitation were made every 2 sec. These data were collected from tissue from a 13-day-old rat in a low-magnesium solution.

experiments were all run in the presence of TTX in order to block both action potentials and action potential dependent synaptic transmission. Bath application of glutamate (50 μM, 60 sec) increased Ca^{2+} levels in almost all SCN cells tested (187 / 190 cells). The average cell showed a Ca^{2+} increase of 118±8% ($n = 190$, $p < .001$) with a duration of 80±2 sec as measured at half of peak. This robust response was completely eliminated in the presence of the broad spectrum GluR antagonist kynurenic acid (1 mM, –4±2%, $n = 12$). The application of 5HT did not have a significant effect on these glutamate-evoked Ca^{2+} transients. In the presence of 5HT (25 μM), glutamate caused a 130±15% ($n = 194$, $p < .001$) increase in Ca^{2+} levels that lasted 74±2 sec. Very similar results were obtained with NMDA in the absence of magnesium. Bath application of NMDA (100 μM, 60 sec) caused robust Ca^{2+} transients in SCN cells that were not altered by 5HT (NMDA: 111±11%, 91 sec, $n = 53$; 5HT + NMDA: 111±11%, 98 sec, $n = 49$). These NMDA-evoked responses were blocked by the presence of AP5 (50 μM, 3±2%, $n = 30$). Finally, we found that 5HT did not modulate these glutamate-evoked Ca^{2+} transients in the day or in the night.

5HT, by Itself, Stimulated Ca^{2+} Transients in a Few Cells

As noted previously, application of 5HT (25 μM, 30-60 sec), by itself, did not have an overall significant effect on resting Ca^{2+} levels (–2±1%, $n = 194$). However, a few cells from this data set (13 / 194) did show a small but detectable response to 5HT (11±2%, $n = 13$). Figure 4 shows Ca^{2+} measurements from two adjacent cells in the SCN; one of these cells showed a repeatable response to 5HT and one did not. 5HT's stimulation of Ca^{2+} appeared to be concentration dependent, with concentrations of 1 μM or less having no measurable effect on Ca^{2+} (Fig. 5, top panel). However at higher concentrations of a longer duration (300 sec), 5HT treatment increased Ca^{2+} in up to 32% of the cells tested. The application of metergoline (10 μM, $n = 61$), TTX (1 μM, $n = 58$), or bicuculline (25 μM, $n = 85$) all significantly ($p < .005$) blocked these 5HT-induced increases in Ca^{2+} (Fig. 5, bottom panel). Finally, we compared the effects of 5HT on Ca^{2+} levels in the day and in the night (Fig. 5, bottom panel). During the day, application of 5HT (100 μM, 300 sec) induced responses in 32 of 100 (32%) cells, whereas during the

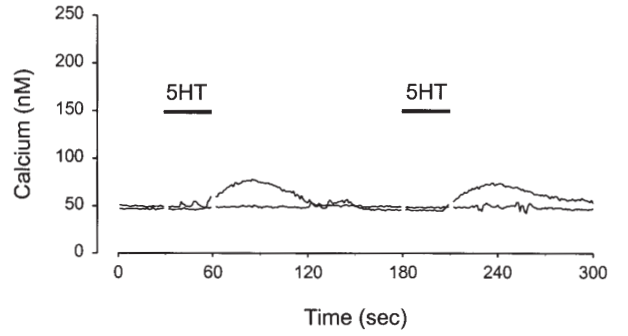

Figure 4. Ca^{2+} measurements from two adjacent cells in the SCN, one of which showed a repeatable response to 5HT (25 μM, 30 sec) and one of which did not. Overall, application of this concentration of 5HT did not have a significant effect on resting Ca^{2+} levels (–2 1%, *n* = 194). However, a few cells (13/194) did show a small but detectable response to 5HT (11 2%, *n* = 13). 5HT's stimulation of Ca^{2+} appeared to be concentration dependent; concentrations lower than 1 μM had no measurable effect, whereas 100 μM 5HT evoked Ca^{2+} transients in about 32% of the cells tested. These data were collected from tissue from a 14-day-old rat.

night, the same treatment induced responses in 18 of 63 (28%) cells. These differences are not significant, and thus we were unable to confirm the previous observation of Mason (1986) that the response of SCN neurons to 5HT varies with the circadian cycle.

DISCUSSION

Information about environmental lighting conditions is conveyed to the SCN, at least in part, via a glutamatergic fiber pathway originating in the retina, known as the RHT. Previous work indicates that 5HT can inhibit this pathway, although the underlying mechanisms are unknown. We became interested in the possibility that 5HT can inhibit the glutamatergic regulation of Ca^{2+} in SCN neurons and, by this mechanism, modulate light-induced phase shifts of the circadian system. To start to examine this hypothesis, optical techniques were used to measure Ca^{2+} levels in SCN cells in a brain slice preparation. First, we found that 5HT produced a reversible and significant inhibition of Ca^{2+} transients evoked by synaptic stimulation. A variety of previous evidence (Colwell and Menaker, 1996; Ebling, 1996) has shown that this synaptic connection is glutamatergic, and we found that GluR antagonists inhibited synaptic-evoked Ca^{2+} transients. We next asked whether 5HT could also inhibit Ca^{2+} transients induced by the direct bath application of

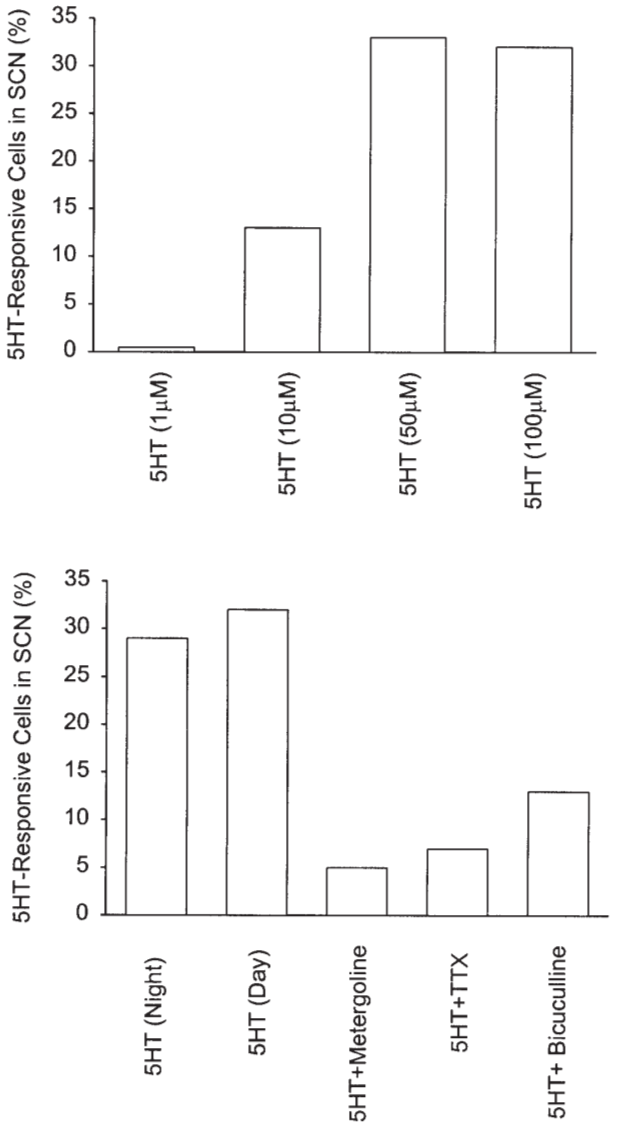

Figure 5. Histogram summarizing data examining percentage of cells in SCN that showed a significant increase in Ca^{2+} in response to 5HT. For any given cell, we defined a detectable response as one in which the change in Ca^{2+} levels was at least twice the standard deviation of our measurement of resting Ca^{2+}. Top panel: Application of 5HT alone (300 sec) produced concentration dependent changes in the percentage of cells that showed a significant increase in Ca^{2+}. Each group contains data from 9 to 100 cells collected from at least 4 animals. Bottom panel: Application of a high concentration of 5HT alone (100 μM, 300 sec) produced a significant response in about one-third of the cells evaluated. There did not appear to be any difference in the response between cells tested in the day or in the night. The application of the 5HT receptor antagonist metergoline (10 μM) significantly ($p < .001$) inhibited these 5HT-induced responses. Finally, the presence of TTX (1 μM), as well as the $GABA_A$ receptor antagonist bicuculline (25 μM) significantly ($p < .001$ and $p < .005$, respectively) inhibited these 5HT-induced increases in Ca^{2+}. Each group contains data from 58 to 100 cells collected from at least 5 animals (10- to 15-day-old rats).

glutamate receptor agonists. We found that 5HT did not alter the magnitude or duration of Ca^{2+} transients evoked by the bath application of glutamate or NMDA in the presence of TTX. We feel that the simplest explanation for these results is that 5HT can act presynaptically at the RHT / SCN synaptic connection to inhibit the release of glutamate. Regardless of the mechanism, our demonstration that 5HT can have a dramatic modulatory action on synaptic-evoked Ca^{2+} transients measured in SCN neurons adds support to the notion that the serotonergic innervation of the SCN may function to regulate environmental input to the circadian system.

The suggestion that 5HT may function presynaptically to modulate synaptic input to the SCN is supported by a number of previous studies. Specifically, several pieces of evidence are consistent with the hypothesis that the $5HT_{1B}$ receptor subtype acts presynaptically on the RHT terminals to gate glutamatergic input to the SCN. 5HT agonists with an affinity for the $5HT_{1B}$ receptor inhibit light-induced phase shifts of locomotor activity rhythm as well as light-induced expression of *c-fos* in the SCN (Pickard et al., 1996). In addition, bilateral enucleation reduces $5HT_{1B}$ receptor binding in the SCN (Pickard et al., 1996). Finally, the $5HT_{1B}$ receptors are located predominantly on axon terminals (Boschert et al., 1994) and act presynaptically to inhibit transmitter release in a number of other brain regions (Maura and Raiteri, 1986; Johnson et al., 1992; Tanaka and North, 1993). Perhaps most relevant, in the superior colliculus, $5HT_{1B}$ receptors are localized on the terminals of retinal afferents and inhibit excitatory input (Waeber and Palacios, 1990; Mooney et al., 1994). Although we did not explore the pharmacology of 5HT's effects, the basic observation that 5HT inhibited synaptic-evoked Ca^{2+} transients but did not alter GluR agonist-evoked responses is at least consistent with a presynaptic mechanism of action. This suggestion is strongly supported by recent work by Pickard and colleagues (1999), who demonstrated that a $5HT_{1B}$ agonist reduces the amplitude of excitatory postsynaptic currents in the hamster SCN without affecting currents generated by application of glutamate.

Besides modulating light input to the SCN, some recent evidence has implicated this serotonergic pathway in mediating activity-induced phase shifts of the SCN circadian oscillator. For example, 5HT release is increased in the SCN region in response to novel running wheel–induced locomotor activity during the day (Dudley et al., 1998). Furthermore, neurotoxic lesions of the serotonergic terminals have been reported to prevent entrainment to daily scheduled activity (Edgar et al., 1997; Marchant et al., 1997). However, other observations appear to cast doubt on any simple interpretation of 5HT's role in these phenomena (e.g., Bobrzynska et al., 1996; Antle et al., 1998). Nevertheless, it is clear that the administration of 5HT receptor agonists causes phase shifts of the SCN circadian oscillator when administered during the subjective day both in vitro (Medanic and Gillette, 1992; Prosser et al., 1993) and in vivo (Tominaga et al., 1992; Edgar et al., 1993; Cutrera et al, 1994). These phase-shifting effects of 5HT are likely to be mediated via activation of $5HT_7$ or $5HT_{1A}$ receptors located directly on SCN cells (e.g., Pickard and Rea, 1997). Although the underlying cellular mechanisms are not well understood, the results from at least one previous study suggested a role for the cAMP / protein kinase A cascade (Prosser et al., 1994). In this study, we found that 5HT administration can increase Ca^{2+} in at least a subpopulation of SCN neurons. This effect of 5HT was concentration dependent and blocked by the broad-spectrum 5HT antagonist, metergoline, which has been shown to block 5HT agonist-induced phase shifts in vitro (Prosser et al., 1993). These observations are consistent with a specific, receptor-mediated mechanism of action for 5HT and raise the possibility that an increase in intracellular Ca^{2+} may be involved in 5HT's phase-shifting actions on the circadian system.

Mintz and coworkers (1997) have reported that the direct application of the GABA antagonist bicuculline into the SCN region prevented phase shifts induced by systemic administration of a 5HT agonist (8-OH-DPAT). They also suggest that 8-OH-DPAT may be acting via a site in the dorsal or medial raphe nuclei. However, it is clear that 5HT and 5HT receptor agonists can cause phase shifts when applied to the SCN brain slice preparation (Medanic and Gillette, 1992; Prosser et al., 1993), so we favor a site of action within the SCN or in the closely surrounding area. Two pieces of data from our current study bear on this issue. The first is our finding that TTX blocks 5HT--induced changes in Ca^{2+}, suggesting that 5HT's actions are mediated through a change in membrane excitability or via a change in action potential dependent synaptic activity. Both membrane excitability and synaptic transmission are inhibited by TTX's action as a blocker of sodium channels. The second observation that bicuculline inhibits 5HT-induced changes in Ca^{2+} leads us to favor the synaptic mechanism. Therefore, our interpretation of this data is that 5HT can act

within the SCN to alter GABAergic activity and, by this mechanism, cause changes in intracellular Ca^{2+}. This interpretation is generally supported by previous work of Kawahara and coworkers (1994), who found evidence that 5HT can inhibit GABA-evoked currents in cultured SCN neurons. We would also like to suggest that this 5HT-induced Ca^{2+} increase plays a role in phase shifts of the SCN circadian oscillator. However, at least one piece of data does not support this simple hypothesis. While we found that TTX blocks 5HT-induced Ca^{2+} transients, Prosser and coworkers report that TTX did not block 5HT-induced phase shifts.

Among the most important consequences of glutamatergic and serotonergic regulation of neurons are the resulting changes in intracellular Ca^{2+} levels (e.g., Mayer and Miller, 1990; Bito et al., 1997). Understanding these changes may be particularly important in the SCN. Photic stimulation results in the release of glutamate from the retinal afferent fibers that innervate the SCN. Many unanswered questions remain as to how the circadian oscillators in the SCN respond to this glutamatergic stimulation. In the simplest case, light causes the release of glutamate that initiates a signal-transduction cascade in SCN neurons, which ultimately results in a phase shift of the circadian system. So what are the next steps in the signal transduction cascade? Activation of GluRs can increase Ca^{2+} both directly by opening ion channels permeable to Ca^{2+} and indirectly through activation of voltage-sensitive Ca^{2+} channels and Ca^{2+}-induced Ca^{2+} release from intracellular stores. This raises the possibility that GluR-induced increase in Ca^{2+} in the SCN is part of the mechanism that underlies light-induced phase shifts. This suggestion is supported by the demonstration that the administration of glutamate increases Ca^{2+} in SCN cells (van den Pol et al., 1992; Tominaga et al., 1994). In addition, there is recent evidence that ryanodine receptors mediate light- and glutamate-induced phase delays of the circadian system (Ding et al., 1998). Similarly, earlier work also indicates that a Ca^{2+} influx is required for light regulation of the circadian oscillator found in the retinas of marine mollusks (e.g., McMahon and Block, 1987; Khalsa et al., 1993; Colwell et al., 1994). Finally, other likely components of this light-induced signal transduction cascade in the SCN include the release of nitric oxide (NO), activation of the Ras/MAP kinase cascade, and, ultimately, changes in gene expression (Ding et al., 1994; Obrietan et al., 1998; Reppert, 1998). In many cell types, the regulation of gene expression, the release of NO, and activation of MAP-kinase signaling pathway are all strongly Ca^{2+} dependent (e.g., Bito et al., 1997). Thus, Ca^{2+} is likely to play a pivotal role in linking GluR activation and other events in this signal transduction cascade. For these reasons, we felt that it was important to investigate 5HT's role in regulating basal- and glutamate-stimulated Ca^{2+} levels. Since the serotonergic system based in the raphe nucleus is thought to play a critical role in determining an organism's arousal state and mood, study of the mechanisms underlying 5HT's actions in the SCN are critical to the development of a mechanistic understanding of the interplay between the arousal, performance, and the circadian system.

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