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Intrinsic Role of Polysialylated Neural Cell Adhesion Molecule in Photic Phase Resetting of the Mammalian Circadian Clock

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The suprachiasmatic nuclei (SCN), the location of the mammalian circadian clock, are one of the few adult brain regions that express the highly polysialylated form of neural cell adhesion molecule (PSA-NCAM). A role for the polysialic acid (PSA) component of PSA-NCAM, which is known to promote tissue plasticity, has been reported for photic entrainment of circadian rhythmicity *in vivo*. The *in vivo* results, however, do not discriminate between PSA acting upstream or downstream of the glutamatergic synapses that convey photic information to the SCN. To address this key issue, we exploited an *in vitro* rat brain slice preparation that retains robust circadian function. As in the intact SCN, PSA levels in the isolated SCN are rhythmic, with higher levels during the early subjective day and lower levels during subjective night. Importantly, bath application of glutamate to SCN slices rapidly and transiently increases PSA levels during both the subjective day and night. Pretreating the slices with endoneuraminidase, which selectively removes PSA from NCAM and thereby prevents this increase, abolishes glutamate- and optic chiasm stimulation-induced phase delays of the SCN circadian neuronal activity rhythm. These results support the hypothesis that PSA expression in the SCN is controlled by both the circadian clock and photic input to the clock and that expression of PSA in the SCN is critical for photic-like phase shifts of the clock. Together, these results establish that such actions of PSA are manifested downstream from presynaptic retinohypothalamic terminals and therefore are intrinsic to the SCN itself.

Key words: circadian rhythms; suprachiasmatic nucleus; polysialic acid; NCAM; phase-shift; glutamate; endoneuraminidase

Introduction

The mammalian suprachiasmatic nuclei (SCN) contain an endogenous circadian clock (Zlomanczuk and Schwartz, 1999). The SCN clock maintains synchrony with the external environment primarily through light input, such that light pulses presented during early night delay the clock, whereas light pulses presented during late night advance the clock. The cellular processes underlying photic phase shifts are complex and incompletely understood but are thought to be initiated by glutamate release from retinohypothalamic (RHT) terminals in the SCN, resulting in postsynaptic increases in intracellular Ca^{2+} and, ultimately, an increased transcription of genes associated with the cellular clock mechanism (Gillette and Tischkau, 1999).

The SCN express a highly polysialylated form of neural cell adhesion molecule (PSA-NCAM) (Glass et al., 1994; Shen et al., 1997, 1999). PSA-NCAM is a negative regulator of cell–cell interactions mediated by a wide variety of receptors (Fujimoto et al., 2001), and this action facilitates important developmental events associated with cell migration and axon guidance (Rutishauser and Landmesser, 1996). Whereas levels of NCAM polysialylation are high during development and generally low in

adult tissues (Chuong and Edelman, 1984; Seki and Arai, 1993), PSA-NCAM continues to be expressed in adult brain regions that have the capacity for structural and/or physiological plasticity. Such regions include the olfactory bulb (Miragall et al., 1990), hippocampus–dentate gyrus (Seki and Arai, 1991, 1993; Le Gal La Salle et al., 1992; Becker et al., 1996; Muller et al., 1996), and hypothalamus (Murakami et al., 1991; Theodosis et al., 1991; Bonfanti et al., 1992; Glass et al., 1994; Lee et al., 1995; Viguie et al., 2001). Evidence for a functional role of polysialic acid (PSA) in brain physiology is suggested by the observations that removing PSA produces ectopic synaptic-like structures (Seki and Rutishauser, 1998), abolishes long-term potentiation (LTP) in the hippocampus (Muller et al., 1996), and prevents structural glial–neuronal reorganizations of the supraoptic nucleus induced by lactation and dehydration (Theodosis et al., 1999).

The expression of PSA-NCAM in the SCN suggests that plasticity in cell interactions is also important to circadian time-keeping regulation. This contention is supported by initial *in vivo* findings that removing PSA impairs circadian rhythm stability (Shen et al., 1997, 2001) and attenuates photic signaling events in the SCN (Glass et al., 2000b). However, *in vivo* it has not been possible to discriminate between effects that PSA might have on intrinsic SCN functions as opposed to events that are involved in providing input to the SCN from the retina. For this purpose, we adopted and validated the *in vitro* rat SCN slice preparation (Gillette et al., 1995) for the study of PSA in circadian photic entrainment in the isolated SCN. Having direct access to the isolated

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SCN permits a more selective activation of SCN photic responses, namely by glutamate and by optic chiasm stimulation. The demonstration that these more central perturbations remain highly dependent on PSA supports the conclusion that PSA is essential for intrinsic aspects of clock entrainment.

Materials and Methods

Brain slice preparation

Coronal brain slices (500 μm) 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 Gillette, 1989; Prosser et al., 1993, 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 (Sigma, St. Louis, MO), pH 7.4.

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 3 M NaCl. Each neuron was recorded for 5 min, and the data were stored for later determination of firing rate using a Data-Wave (Longmont, CO) system. Typically, four to seven cells were recorded during each hour. These firing rates were then used 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 15 min, the time of symmetrically highest activity.

Experimental protocols

Drug treatments. Glutamate (1 mM) was bath applied to the brain slices beginning at either zeitgeber time 6 (ZT 6) (ZT 0 is lights-on in the donor colony) or ZT 14 on the first day *in vitro* by stopping the perfusion and replacing the medium in the slice chamber with medium containing glutamate. After 10 min, the normal medium was reintroduced into the slice chamber, and perfusion was resumed. Another group of slices was treated in a similar manner at ZT 6 with a combination of glutamate antagonists. For blocking experiments using endoneuraminidase (endo N), at ZT 12 on the first day *in vitro*, the bathing medium was replaced with medium containing endo N diluted to 20–100 U/ml. The endo N-containing medium (total volume, 15 ml; chamber volume, <3 ml) was continually recirculated between the slice chamber and a reservoir in which it was rewarmed and reoxygenated. After 2 hr, the recirculation was stopped, and the medium in the slice chamber was replaced with medium containing endo N and glutamate. After 10 min, this medium was exchanged for medium containing endo N, and the recirculating perfusion was reinstated. After 50 additional minutes, this medium was replaced with normal medium, and normal perfusion was resumed. Control experiments were conducted to determine whether the recirculation procedure (using normal medium) altered the circadian rhythm of neuronal activity. No effect was seen (data not shown). Therefore, the times-of-peak for drug-treated slices were compared with the mean time-of-peak for untreated slices (ZT 6.0 \pm 0.3; $n = 3$) to determine the amount of phase shift induced by the treatment. Chemicals used in the study were L-glutamate, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonopentanoic acid (AP-5) (all from Sigma), and endo N (U. Rutishauser).

Optic chiasm stimulation. Optic chiasm stimulation was performed as described previously (Prosser, 1998a). Briefly, a bipolar, blunt-cut, insulated platinum-iridium electrode was positioned in the optic chiasm ventrolateral to the SCN. Voltage (10 Hz, 10V, 3 msec duration) was applied for 10 min.

Daily rhythm of PSA expression. To determine whether a daily rhythm in PSA expression persists *in vitro*, SCN brain slices were prepared during the subjective day and maintained as described above. Slices were then removed from the slice chamber at 4 different time points (ZT 6, ZT 12, ZT 18, and ZT 24), and the optic chiasm was removed. The remaining tissue, consisting almost entirely of SCN tissue, was frozen on dry ice.

Samples consisting of single SCN slices were then stored at -80°C . Western blot analyses of tissue PSA content were subsequently performed on the tissue extracts as described below.

Immunological analyses

Immunoblot analyses. Brain slices were prepared as described above, trimmed to contain only the SCN and underlying optic chiasm. Time of slice preparation was varied to equalize the time *in vitro* as much as possible. Slices were maintained in the slice chamber for at least 1 hr after slice preparation. At the appropriate time, each slice was removed and rapidly frozen on dry ice, and the optic chiasm was removed using a single-edged razor blade. The remaining tissue was then weighed and stored at -80°C . The samples were weighed and sonicated in 20 vol of 0.01 M PBS, pH 7.4, containing Nonidet P-40 (Sigma) and aprotinin (20 $\mu\text{g}/\text{ml}$; Sigma). The tissue homogenates were centrifuged at 13,600 $\times g$ at 4°C for 5 min. A 1 μl aliquot of the supernatant from each sample was used for protein determination using a micro BCA protein assay reagent kit (Pierce, Rockford, IL). The remainder of the supernatant was diluted with 1 vol of 1.6 ml of glycerol, 3.0 ml of 10% SDS, 0.8 ml of 2,6-mercaptoethanol, and 0.4 ml of 0.05% bromophenol blue, boiled for 3 min, and stored at -70°C until immunoblot analysis. An equal amount of total protein (20 μg) from each sample was loaded on a 5% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Arlington Heights, IL) at 35 V for 18 hr, and PSA was detected by incubation with a characterized mouse monoclonal antibody 5A5 (IgM) against α -2-8-linked PSA of NCAM (Acheson et al., 1991), followed by a peroxidase-conjugated second antibody, and then reacting the bound peroxidase with chemiluminescent substrate (ECL; Amersham Biosciences). Chemiluminescence was detected by exposure to x-ray film (Hyperfilm-ECL; Amersham Biosciences). The exposed signals on the film were scanned and then quantified using Gel-Pro software (Media Cybernetics, Silver Spring, MD).

Immunohistochemical procedures. Brain slices were prepared and maintained for at least 1 hr after slice preparation. At the appropriate time, each slice was removed from the slice chamber and placed in 2% paraformaldehyde for 3 hr at 4°C. The 500- μm -thick slices were then placed in 0.1 M PBS and kept at 4°C. Subsequently, the slices were glued to a cutting block with cyanoacrylate, and vibratome sections (60 μm thick) were cut, washed in 0.1% PBS, and incubated with 1% H₂O₂ to inactivate endogenous peroxidase. Sections then were sequentially incubated in the following solutions with buffer washes in between: (1) 5A5 primary antibody diluted 1:500 with incubation buffer (50.0 mM Tris-buffered saline, pH 7.6, containing 0.9% NaCl, 0.1% bovine serum albumin, and 0.1% Triton X-100) at 4°C overnight; (2) secondary antibody conjugated with peroxidase (goat anti-mouse IgM diluted 1:200; Vector Laboratories, Burlingame, CA) at room temperature for 1 hr; and (3) 0.02% diaminobenzidine with 0.009% H₂O₂ at room temperature for 5–10 min. Stained sections were floated on to a gel-coated slide and mounted with coverslips with Permount (Fisher Scientific, Houston, TX).

endo N preparation

Recombinant endo N was expressed by isopropylthioglycoside induction of pREP4-repressed M15 cells containing a QE60 (catalog #33603; Qiagen, Hilden, Germany) plasmid encoding endo N (Vimr et al., 1984) plus a 6xHis-tag. After 3 hr, the cell pellet was lysed in 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 10 mM imidazole plus !X Protease Inhibitor (Boehringer Mannheim, Mannheim, Germany), treated with lysozyme DNase and RNase on ice, and centrifuged to remove cell debris. The endo N was absorbed to a column of Ni-NTA-coupled Superflow resin (Qiagen) and eluted with lysate buffer containing 250 mM imidazole. Fractions containing endo N, as determined by SDS-PAGE, were diluted with an equal volume of glycerol and dialyzed at 4°C against PBS, pH 8.0, and 50% glycerol. The specific activity of the purified endo N (dilutions containing 2.5–160 ng) was determined using 1 μg of PSA-NCAM substrate obtained from embryonic chick brain and a 30 min reaction at 37°C. After the reaction, the protein was adsorbed to nitrocellulose and immunostained for residual PSA using the 5A5 IgM monoclonal anti-PSA and goat anti-mouse IgM coupled to alkaline phosphatase. One unit of activity was defined as the amount of enzyme required to remove

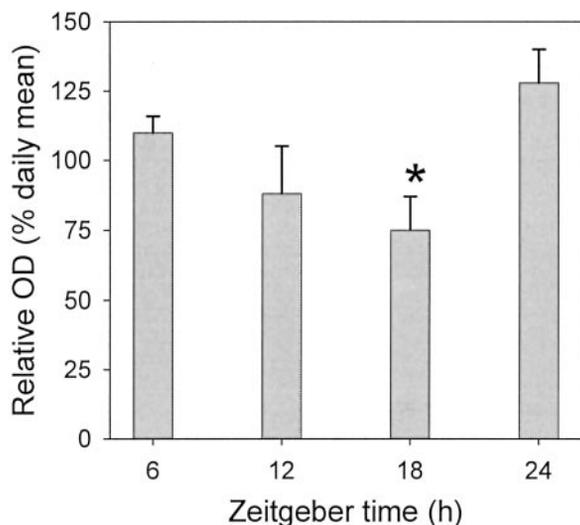


Figure 1. Circadian variation in the tissue content of PSA in the SCN at different times of the circadian cycle assessed by immunoblot assay. *OD*, Optic density. $n = 3$ per time point. Error bars are SEM; * $p < 0.05$ versus ZT 24 level.

one-half of the PSA staining. The purified endo N had no detectable protease activity or toxicity in 3 d cultures of primary neurons.

Statistical analyses

Differences in time of peak neuronal activity were assessed using Student's *t* test or ANOVA analyses when appropriate. Differences in PSA expression were assessed by one-way ANOVA, followed by Tukey's multiple comparison *post hoc* test. In all cases, the level of significance was set at $p < 0.05$.

Results

The circadian rhythm of SCN PSA expression persists *in vitro*

PSA levels in the SCN slice preparation exhibited a distinct and reproducible rhythm, similar to that seen *in vivo*, with higher levels during the subjective day and lower levels at night (Fig. 1). The tissue content of PSA at ZT 18 was $75.0 \pm 0.1\%$ of the daily mean ($p < 0.05$ vs ZT 24 level).

endo N removes SCN PSA *in vitro*

endo N selectively cleaves PSA from its NCAM carrier (Vimr et al., 1984; Acheson et al., 1991), and, for the following studies, it is critical that this treatment is effective with the SCN tissue slices. Using immunohistochemical and immunoblot procedures, it was found that the addition of endo N into the perfusion medium, as described below in the photic phase-shifting experiments, completely removed immunoreactive PSA from the SCN slice within 2 hr. Shown in Figure 2 are representative SCN slices, corresponding to subjective daytime, after immunostaining for PSA. Evident from the control slice is the strong pattern of PSA expression throughout the SCN, demarcating the nucleus from the surrounding hypothalamus and optic chiasm. In contrast, PSA staining was absent in the endo N-treated slice. The endo N removal of PSA from the slice was confirmed by immunoblot assay (data not shown).

Glutamate induces SCN PSA expression *in vitro*

Having determined that PSA in the SCN *in vitro* is low during the subjective night, it was important to determine whether glutamate treatment affects PSA levels in the SCN at a time when glutamate has been shown to induce 3–4 hr phase delays (ZT 14) (Ding et al., 1994; Forrest and Prosser, 2000). In the present

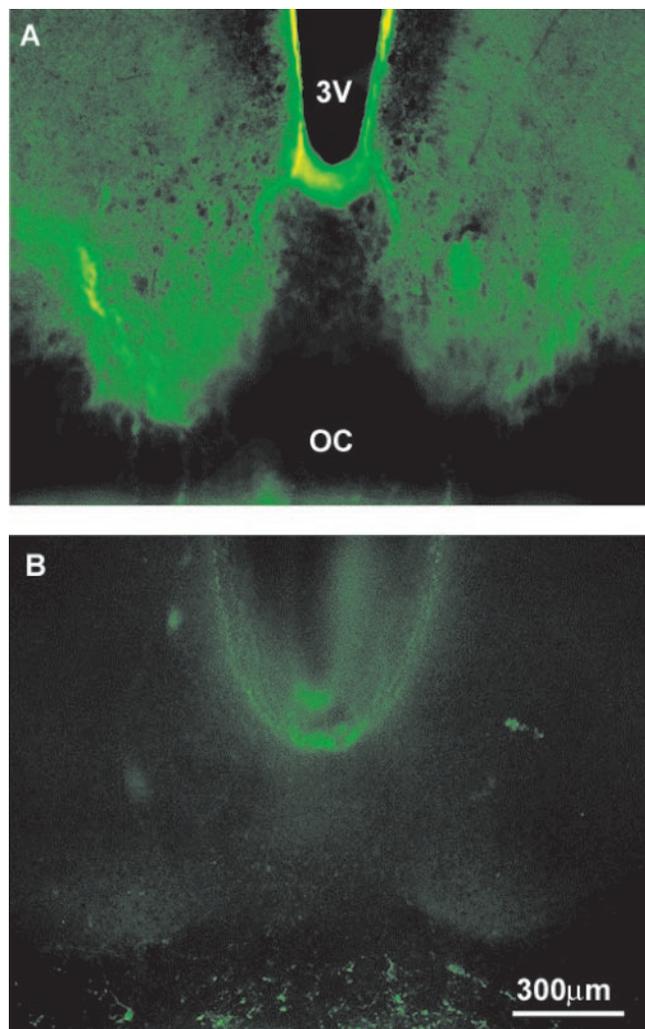


Figure 2. Immunohistochemical staining for PSA in the SCN at ZT 6. The PSA (green labeling) is clearly seen throughout the SCN under normal *in vitro* conditions (A) but is absent from the SCN after treatment with endo N (B). 3V, Third ventricle; OC, optic chiasm.

experiments, slices treated for 10 min with glutamate (1 mM) at ZT 14 showed a significant increase in PSA expression to 200% of the pretreatment level (Fig. 3) ($p < 0.05$ vs pretreatment level). This increase occurred within 30 min of the glutamate application and lasted ~2 hr, after which PSA expression decreased to levels measured in untreated slices. Glutamate application at ZT 6 for 10 min, when *in vitro* PSA levels are already high, similarly increased PSA levels 30 min later to $173 \pm 8\%$ ($n = 3$) of controls ($p < 0.01$ vs untreated slices). Conversely, a 10 min application of a mixture of glutamate antagonists (CNQX and AP-5) at ZT 6 did not significantly change PSA expression *in vitro* (data not shown).

endo N blocks glutamate induction of SCN PSA *in vitro*

Using the same procedure as in the glutamate experiment, SCN slices were exposed to endo N for 3 hr beginning at ZT 12. At ZT 14, they were given a 10 min pulse of glutamate (1 mM). As shown in Figure 3, with endo N present, no PSA was detectable from ZT 14 to ZT 17, confirming that the concentration of enzyme used in these trials is sufficient to block the stimulated increase in PSA that occurs in response to a potentially phase-resetting application of glutamate.

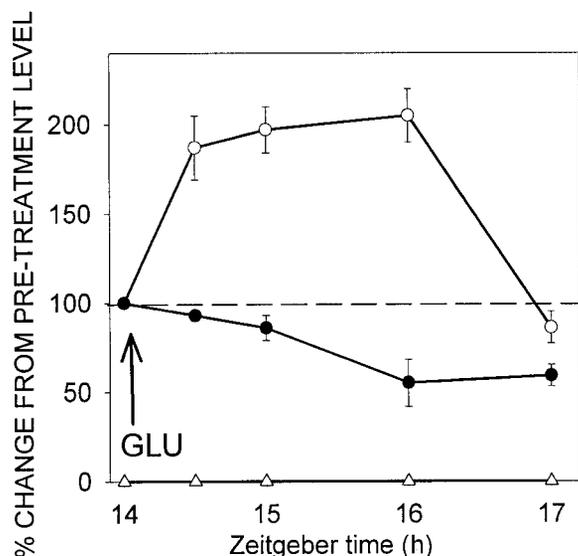


Figure 3. Glutamate acutely stimulates PSA expression in the SCN slice. A 10 min application of glutamate induced an ~200% increase in total SCN PSA content within 30 min of treatment. The PSA levels in untreated controls exhibited a gradual decrease throughout the subjective night. Pretreatment of slices with endo N prevented any glutamate-induced stimulation of PSA. Each time point is an average of three samples. *Open circles*, Glutamate-treated tissue; *filled circles*, no treatment; *open triangles*, glutamate plus endo N-treated tissue.

endo N blocks phase delays induced by *in vitro* electrical stimulation of the optic chiasm

Electrical stimulation of the optic chiasm (OCS) was used to directly confirm an essential role for PSA in photic-like phase-resetting events in the SCN slice. The OCS procedure mimics photic activation of the SCN and induces phase shifts *in vitro* that are similar in magnitude and direction to those induced by glutamate (Ding et al., 1994; Forrest and Prosser, 2000). This is believed to occur because electrical stimulation induces release of the retinohypothalamic neurotransmitter glutamate from the retinal ganglion cell terminals (Liou et al., 1986). Depicted in Figure 4, *A* and *B*, are representative experiments showing the neuronal activity rhythm from an untreated slice and a 3.5 hr phase delay in the neuronal activity rhythm induced by 10 min of OCS at ZT 14. When OCS was combined with 3 hr endo N treatment from ZT 12 to ZT 15, the phase delays were completely blocked (Fig. 4*C*). In contrast, OCS applied to SCN slices in the presence of inactive (boiled) endo N continued to induce robust phase delays (Fig. 4*D*). Slices receiving a 3 hr incubation with endo N alone showed no phase-resetting response (data not shown). The results of these experiments are summarized graphically in Figure 5.

endo N blocks glutamate-induced phase delays *in vitro*

In a complementary experiment, the effects of endo N on the phase-resetting action of the RHT transmitter glutamate were assessed. This approach was also used to explore a post-RHT mechanism of action of PSA. Depicted in Figure 6*A* is a representative experiment showing the phase-delaying effect of a 10 min pulse of glutamate delivered to the slice at ZT 14. As with OCS, this treatment induced a robust phase delay of 3.5 hr in the circadian rhythm of neuronal activity compared with untreated slices. Conversely, in slices incubated with endo N for 3 hr from ZT 12 to ZT 15, the phase-resetting effect of glutamate is totally abolished (Fig. 6*B*). These results are summarized graphically in Figure 5.

Discussion

The demonstration that PSA expression in the SCN slice fluctuates with a similar pattern as that observed *in vivo* (Glass et al., 2001) attests to the SCN slice being a physiologically viable model for studying PSA function in the circadian clock. Using this system, the present results point to an essential role for PSA-NCAM in photic regulation of the mammalian circadian clock. Notably, when PSA is enzymatically removed from the SCN slice, the phase-resetting effects of photic-like signaling stimuli (electrical stimulation of the optic chiasm and application of glutamate) are blocked. This functional link between PSA and photic entrainment is consistent with the correlative observation that glutamate-induced phase delays occur in conjunction with a dramatic upregulation of SCN PSA expression. These results support previous *in vivo* studies pointing to a role for PSA in photic entrainment (Shen et al., 1997; Glass et al., 2000b). Most importantly, because removing PSA blocks the direct action of glutamate in the SCN, PSA must act downstream from presynaptic RHT terminals and therefore is intrinsic to SCN function.

Pattern and kinetics of PSA expression in the SCN slice

Analyses of PSA immunostaining in the SCN of a number of species, including mouse and Siberian and Syrian hamsters, have revealed that PSA expression occurs throughout the rostrocaudal extent of the SCN, with denser staining in the ventrolateral (“core”) compared with the dorsomedial (“shell”) region (Glass et al., 1994, 2001; Shen et al., 1997). Whether the more intense staining in the core region reflects an association between PSA and retinorecipient units, which are found predominantly in the ventrolateral region (Morin, 1994), or whether it is a consequence of the higher cell density in this part of the SCN (van den Pol, 1980) is not known. However, in the hamster, intense PSA immunoreactivity is closely associated with cells expressing light-induced Fos protein and with calbindin D_{128K} cells, which receive RHT input (Bryant et al., 2000; Glass et al., 2001). From the present immunohistochemical analysis of vibratome sections of rat SCN slices, the pattern of PSA expression is similar to that observed in the SCN of the other rodent species, indicative of a similar generalized association between PSA-expressing elements and retinorecipient units.

Within the SCN, PSA expression is regulated by the circadian pacemaker: under constant dark conditions, PSA levels peak during subjective day and are lowest during subjective night *in vivo* (Glass et al., 2001). Here we show that a similar pattern is expressed virtually unchanged *in vitro*. This indicates that circadian control of PSA expression involves processes intrinsic to the SCN and does not rely on clock outputs indirectly feeding back onto the SCN.

Expression of PSA in the SCN is also strongly influenced by photic input, because light pulses presented to hamsters during late subjective night rapidly increase the normally low SCN PSA content to high daytime levels, as assessed using quantitative immunoblot analysis (Glass et al., 2000a). This response was specific to the SCN, because PSA levels in other regions were unaffected by light. Here we show that photic-like stimulation also increases PSA expression in the SCN *in vitro* using the same immunoblot analysis. In these trials, application of glutamate at ZT 14 increased PSA levels with a time course remarkably similar to that seen *in vivo* [i.e., PSA content increased within 30 min of stimulation in both cases and remained elevated for 1 hr (*in vivo*) or 2 hr (*in vitro*) before returning to unstimulated levels]. Interestingly, glutamate applied during the subjective day increased PSA levels to a comparable degree. The fact that PSA content of the

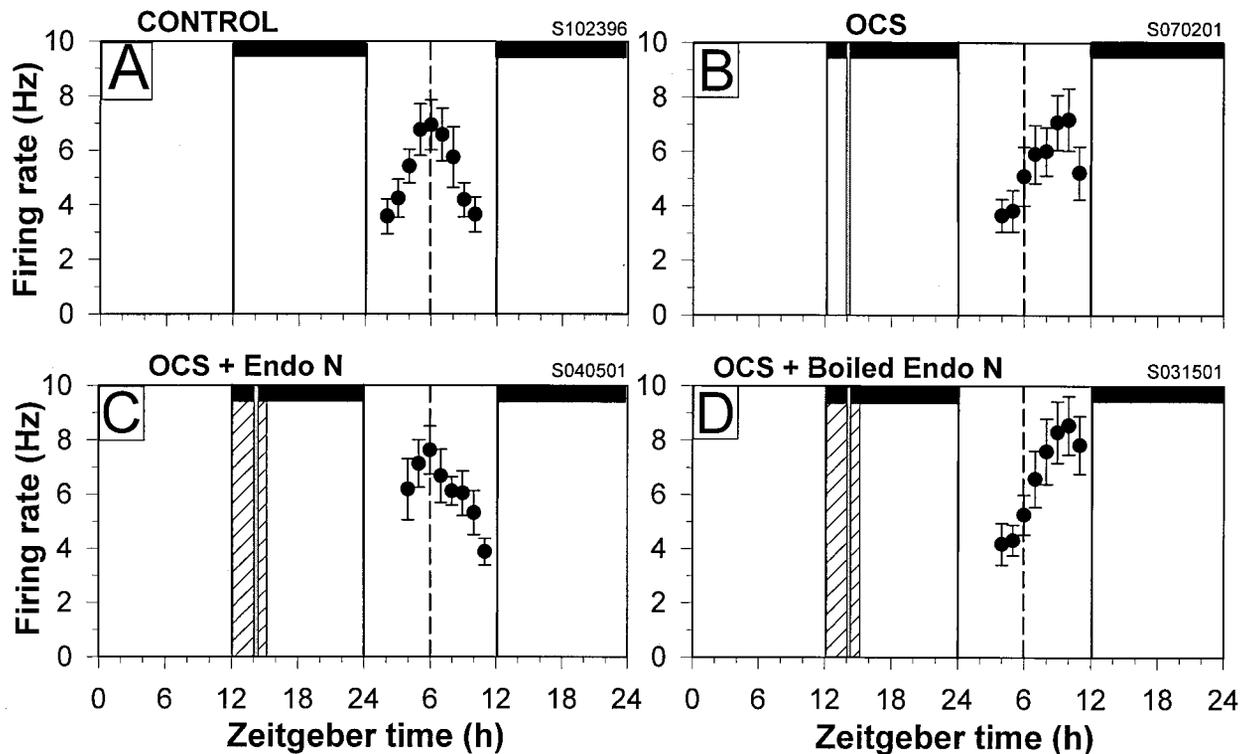


Figure 4. endo N blocks *in vitro* phase shifts induced by optic chiasm stimulation. Shown are representative single-unit activity recordings from SCN slices after the following treatments: *A*, no treatment, showing the normal peak in activity at ZT 6; *B*, optic chiasm stimulation at ZT 14, showing a 3.5 hr phase delay in activity; *C*, optic chiasm stimulation plus endo N, showing no phase shift; and *D*, optic chiasm stimulation plus inactive endo N, showing a 3.5 hr phase delay. Horizontal bars, Time of lights-off in the animal colony; vertical bars, times of treatment; dotted lines, mean time of peak neuronal activity in control slices.

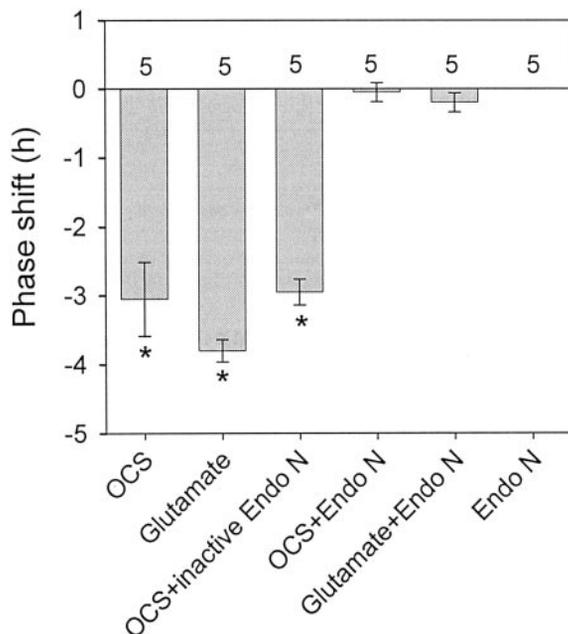


Figure 5. Histogram plot summarizing the results of phase-shifting experiments. Shown are the mean \pm SEM phase shifts induced by the different experimental conditions relative to the mean time-of-peak in control slices (ZT 6.0 ± 0.3 ; $n = 3$). Numbers below each bar indicate the number of replicate experiments. * $p < 0.05$ versus untreated slices.

SCN increases after glutamate application *in vitro* provides strong evidence that the processes mediating photic induction of PSA are contained within the SCN. The rapid time course for the increase in PSA content suggests further that the increase in SCN

PSA is the result of a post-transcriptional event in PSA synthesis, most likely a change in the activity of polysialyltransferase, the enzyme that catalyzes PSA synthesis and attachment to its NCAM carrier. Such a change in activation has in fact been observed in the response of neurons when they establish connections with their targets (Bruses and Rutishauser, 1988). Our results showing daytime as well as nighttime increases in PSA levels in response to glutamate, coupled with the inability of glutamate antagonists to decrease daytime PSA expression, suggests that the *in vitro* rhythm in PSA expression is not being driven by a rhythm in glutamate release in this deafferented preparation. Thus, at least *in vitro*, endogenous control of PSA expression must involve nonglutamatergic mechanisms.

PSA and photic phase resetting

With respect to cellular mechanisms, the most salient finding of this study was that transient removal of PSA from the SCN completely blocked the phase-shifting effects of glutamate. This blockade indicates that the effects of PSA occur downstream from the presynaptic RHT terminals; that is, they are central to the regulation of the SCN clock rather than being derivative of effects upstream from the receipt of photic information. This is a fundamental distinction, because PSA is known to have a variety of effects on axon sprouting and synaptogenesis in CNS target regions (Bruses and Rutishauser, 2001). It is noteworthy that daytime glutamate induction of PSA (shown here) is not accompanied by pacemaker resetting (Ding et al., 1994). This indicates that additional processes characteristic of the nocturnal SCN must also be needed for photic phase shifting to occur.

The involvement of PSA in fundamental nervous system physiology has been noted in other studies. For example, PSA is

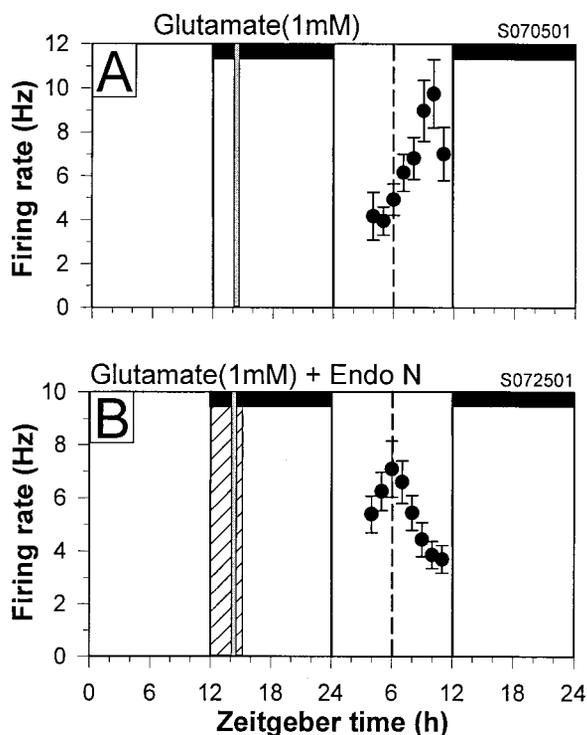


Figure 6. endo N blocks *in vitro* phase shifts induced by glutamate application to the SCN. Shown are representative single-unit activity recordings from SCN slices after the following treatments: *A*, glutamate (1 mM) at ZT14, showing a 3.5 hr delay in neuronal activity; and *B*, glutamate (1 mM) plus endo N, showing no phase shift. For details, see Figure 4 legend.

essential for normal hypothalamo-neurohypophyseal activities during lactation and dehydration (Theodosis et al., 1999), and there is a correlation between PSA expression and LTP-associated morphological reorganizations of adult hippocampus (Regan and Fox, 1995; Muller et al., 1996; Cremer et al., 2000; Ronn et al., 2000). With respect to synaptic mechanisms, it is notable that the SCN exhibits LTP and long-term depression (Nishikawa et al., 1995; van den Pol et al., 1996), and we showed that some PSA-NCAM is also closely associated with synaptic complexes in the SCN (Shen et al., 1999). The cellular mechanisms proposed for PSA involvement in these two systems are very different, namely modulating synaptic efficacy of CA1 neurons versus facilitating changes in neuronal–glial cell interactions that control hormone release. Interestingly, either of these mechanisms could be relevant to the SCN, in which changes in both neuronal connectivity and glial cell influences have been noted (Lavialle and Serviere, 1993; Colwell, 2000; Harley et al., 2001).

Other studies on the role of PSA in learning and memory suggest that it may enhance the ability of the neurotrophin brain-derived neurotrophic factor (BDNF) to interact with its Trk β receptor. Electrically stimulated hippocampal slices from PSA-NCAM knock-out mice and hippocampal slices treated with endo N exhibit reduced amounts of Trk β phosphorylation, which is an indicator of BDNF activity. Notably, treatment with exogenous BDNF reverses the loss of LTP and decreases Trk β phosphorylation exhibited by these slices (Muller et al., 2000). A similar interaction between PSA and BDNF has been seen with respect to cortical cell differentiation (Vutskits et al., 2001). Although it is not known whether PSA enhances BDNF activity in the SCN, it is noteworthy that recent data point to a role for BDNF in regulating photic shifting of the SCN clock (Liang et al., 1998, 2000).

Although removing PSA blocks the effects of glutamate, slices treated with endo N retain their normal circadian cyclicality, as assessed the day after enzyme treatment, with no significant change in rhythm phase or amplitude. Therefore, endo N treatment is not injurious to the neural clock apparatus, and removing PSA, at least temporarily, does not interfere with ongoing pacemaker activity. This latter observation indicates that the role of PSA-NCAM in the SCN may be restricted to clock entrainment and not to pacemaker signal generation per se.

In other studies, we found that PSA also influences nonphotic responses of the SCN (Fedorkova et al., 2002). Together with the present findings, this suggests that PSA expression in the SCN is requisite for all phase-shifting responses of the circadian clock (both photic and nonphotic) and therefore represents a central component of clock plasticity. Such a role, however, will likely prove to be complex, in that the nonphotic phase-resetting effect of the serotonin agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin is significantly enhanced, rather than blocked, by endo N treatment (Fedorkova et al., 2002). One explanation for these opposite effects is that PSA enhances the response to glutamate, and, because glutamate is known to block serotonergic phase shifts *in vitro* (Prosser, 2001), removal of PSA could in principle reduce the glutamatergic inhibition of serotonergic phase-resetting actions. As a consequence, high levels of PSA during the day may normally act as a physiological dampener of daytime phase-resetting effects of nonphotic stimuli.

In conclusion, PSA expression in the acute SCN slice is regulated by the circadian pacemaker and by the photic-like influence of glutamate. Furthermore, enzymatic removal of PSA from the isolated SCN blocks the phase-resetting effects of glutamate and optic chiasm stimulation. Collectively, these data point to a critical role for PSA in the photic regulation of mammalian circadian clock phase.

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