

Circadian Photic Regulation of Melatonin Receptor Density in Rat Suprachiasmatic Nuclei: Comparison With Light Induction of Fos-Related Protein

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High-affinity melatonin receptors are present in rat suprachiasmatic nuclei (SCN), and their density exhibits a daily rhythm regulated by the light/dark cycle. In this study we demonstrate that the light regulation of these receptors depends on a circadian mechanism. Pinealectomized rats kept in constant darkness were subjected to 1-hr light pulses delivered across the circadian cycle. The density of melatonin receptors was significantly increased when photic exposure was performed during subjective night, and not different from control animals kept in darkness when the light pulse was applied during subjective day. The protein product (Fos) of the immediate early gene c-fos studied in the same paradigm showed globally the same circadian sensitivity phase. These results clearly show that, although the rhythmic appearance of melatonin receptor density in SCN follows and is directly regulated by the standard light/dark cycle, this light regulation is not passive. As is the case with Fos-like protein, it is only during a precise phase of the circadian cycle that light is able to regulate the density of melatonin receptors in SCN.

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Key words: melatonin receptors, circadian rhythm, light induction, immediate early gene, constant darkness

INTRODUCTION

Mammalian suprachiasmatic nuclei (SCN) contain the circadian biological clock that controls a wide array of behavioral and physiological rhythms including locomotion, sleep-wake cycle, thermoregulation, cardiovascular function, and many endocrine processes (reviewed in Moore, 1983; Turek, 1985; Meijer and Rietveld, 1989). Among these are the night synthesis and secretion of the pineal hormone melatonin (Klein, 1979). High-affinity melatonin receptors are present in SCN of many

species of mammals (Vanecek et al., 1987; Weaver et al., 1991; Morgan et al., 1993; Stankov et al., 1993; Masson-Pévet et al., 1994; Reppert et al., 1994), and some evidence indicates that melatonin might feed back on SCN to modulate circadian patterns of activity (Redman et al., 1983; Cassone, 1990; Kirsch et al., 1993). Receptor autoradiography has revealed that the density of melatonin receptors in rat SCN exhibits diurnal variations, with low levels during the night (Gauer et al., 1993; Tenn and Niles, 1993). These day/night variations are independent of circulating melatonin concentrations, and are directly induced by the light/dark cycle (Gauer et al., 1994a). Moreover, the decrease in melatonin receptor density during the night is suppressed by exposing the animals to 1 hr of light (Gauer et al., 1994a), and this light effect is mediated through activation of N-methyl-D-aspartic acid (NMDA) receptors (Gauer et al., 1994b). Exposure of rats to light pulses during the night is also known to increase immunoreactivity for the protein product of the immediate-early gene c-fos within SCN (Aronin et al., 1990; Earnest et al., 1990; Rusak et al., 1990), through activation of NMDA receptors (Abe et al., 1991). This effect has been shown to be dependent on periods of sensitivity to light only during subjective night (Rusak et al., 1992; Earnest and Olschowka, 1993). The aim of the present study was to determine whether the day/night variation in melatonin receptor density is passively dependent on the light/dark cycle or, as with Fos, it depends on a circadian phase of light sensitivity. To answer this question, the density of melatonin receptors was measured in SCN of rats kept in constant darkness and subjected to light pulses delivered across the circa-

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dian cycle. As melatonin is known to autoregulate its own receptors in SCN (Gauer et al., 1993), these experiments were performed in pinealectomized animals to eliminate the presence of this hormone. To compare the results obtained with a known circadian light-sensitive system, Fos immunoreactivity was concomitantly analyzed in the same paradigm.

MATERIALS AND METHODS

Animals and Protocols

Male Wistar rats (200–250 g) (Iffa Credo, l'Arbresle, France) were maintained under a 12-hr light/12-hr dark cycle (12L/12D), with lights on at 07.00 hr (300–550 lux) for 2 weeks prior to experimentation. A dim red light (<1 lux) was always present during the dark period of the cycle, and food and water were provided ad libitum.

Experiment 1: Effect of 1-hr light pulses on density of SCN melatonin receptors. Rats ($n = 96$) were pinealectomized under pentobarbital anesthesia (35 mg/kg, i.p., Sanofi, Libourne, France) 3 days prior to experimentation and kept afterwards in constant darkness (DD) (with a permanent dim red light). They were sacrificed at eight different circadian times (CT) relative to predicted activity onset (CT12), four during subjective day (CT1, CT4, CT8, and CT10), and four during subjective night (CT13, CT16, CT19, and CT22). For each CT, 12 animals were sacrificed: 6 in darkness and 6 after a 1-hr-long light pulse.

Experiment 2: Effect of 1-hr light pulses on Fos-immunoreactive (Fos-ir) cells in ventrolateral SCN. The paradigm used was the same as described in experiment 1. Three animals were sacrificed at the same eight time points (CT2, CT5, CT9, CT11, CT14, CT17, CT20, and CT23), 1 in darkness and 2 after a 1-hr-long light pulse. Moreover, to make sure that pinealectomy (and thus absence of melatonin) did not interfere with light-induced Fos expression, 3 intact animals were also used at both CT2 and CT5, 1 sacrificed in darkness and 2 after light exposure.

In Vitro Autoradiography Procedure and Data Analysis

Animals were sacrificed by decapitation. Brains were rapidly removed, frozen in isopentane maintained at -30°C , and stored at -20°C – -30°C until sectioning. Serial coronal sections (20- μm thick) of region containing SCN were cut on a Reichert-Jung cryostat (Leica, Lyon-Bron, France), thaw-mounted onto gelatin-coated slides, and kept at -30°C until use. Sections were preincubated at 4°C for 15 min in 100 mM Tris buffer containing 4 mM CaCl_2 , pH 7.4, and then incubated in the same buffer containing various concentrations of 2- ^{125}I -

melatonin (synthesized according to the method of Vakuri et al. (1984) and purified by high performance liquid chromatography [HPLC]). Sections were washed twice for 30 sec in assay buffer, followed by a 30-sec wash in distilled water at 4°C with agitation. Hyperfilm (^3H , Amersham, Les Ulis, France) were placed on the air-dried sections for 10 days in the presence of 20- μm thick ^{125}I microscale standards (Amersham). Quantitative analysis of autoradiograms was performed using the computerized analysis system Biocom-program RAG 200. Optical density of autoradiograms was converted into fmol/mg polymer using the microscale standards, and the resulting data were then converted into fmol/mg protein as described by Nazarali et al. (1989). Specific 2- ^{125}I -melatonin binding (binding to receptors only) was determined as the difference between total and nonspecific binding (not displaced in the presence of 1 μM cold melatonin). The saturation curve was analyzed by the equation

$$Y = AX/B + X, \text{ where } A = B_{\text{max}} \text{ and } B = K_d \text{ (Graph Pad, Graph Pad Inc., San Diego, CA).}$$

Immunohistochemistry Procedures

Animals were deeply anesthetized with pentobarbital (35 mg/kg, i.p.) and perfused through the ascending aorta with 50 ml of NaCl (0.9%) solution, followed by 250 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4 at room temperature). Brains were rapidly dissected out, then postfixed for 12 hr in the same fixative at 4°C , and 50- μm coronal sections were made on a vibratome in phosphate-buffered saline (PBS; 0.1 M, pH 7.4). Free-floating sections were incubated for 12 hr at 4°C in Fos polyclonal antibody raised in sheep (Cambridge Research Biochemicals, Wilmington, DE), diluted at 1/5,000 in PBS 0.1 M containing 0.5% triton-X100 and 0.25% gelatin (PBST). After several rinses in PBS, sections were incubated for 1 hr at room temperature in a solution containing biotinylated anti-sheep antiserum made in rabbit, diluted 1/500 in PBST. After multiple rinses with PBS, sections were incubated for 1 hr at room temperature in an avidin-biotin-peroxidase complex (Vectastain ABC-reagent, Biosys SA, Compiegne, France) diluted in PBST. The tissue was washed repeatedly with PBS and then treated with a chromagen solution consisting of 0.025% diaminobenzidine, 0.5% nickel ammonium sulfate, and 0.015% hydrogen peroxide in TRIS-HCl buffer (0.05 M, pH 7.4) at room temperature. The nickel-enhanced diaminobenzidine reaction produced a black-blue product.

The number of Fos-ir nuclei was counted manually in all ventrolateral subdivisions of SCN, irrespective of intensity of staining.

TABLE I. 2-¹²⁵I-Melatonin-Specific Binding in SCN of Pinealectomized Rats Kept in Constant Darkness (DD) or Subjected to One Hour-Long Light Pulse (DD + 1HL) Delivered at Eight Circadian Times (CT)

Circadian time	Specific binding (fmol/mg protein)	
	DD	DD + 1HL
CT2	5.16 ± 0.10	4.84 ± 0.17
CT5	4.51 ± 0.31	4.31 ± 0.14
CT9	5.56 ± 0.16	5.37 ± 0.36
CT11	4.85 ± 0.39	4.13 ± 0.22
CT14	4.51 ± 0.22	5.88 ± 0.25*
CT17	5.40 ± 0.26	6.90 ± 0.53*
CT20	5.45 ± 0.24	7.21 ± 0.27*
CT23	4.67 ± 0.18	5.39 ± 0.14**

* $P < 0.01$, when compared to respective control group killed in DD.

** $P < 0.05$, when compared to respective control group killed in DD.

RESULTS

Experiment 1

In pinealectomized animals kept in DD, 2-¹²⁵I-melatonin binding in SCN was significantly increased after a 1-hr light pulse when the photic exposure was performed during subjective night at the four CTs studied (Table I). When the light pulse was applied during subjective day, no difference in specific binding was observed between light exposed and control animals (Table I, Fig. 1). Saturation study performed at CT17 with or without a light pulse revealed values of B_{\max} of 8.53 ± 0.79 fmol/mg protein and 6.43 ± 0.36 fmol/mg protein, respectively ($P < 0.05$), and K_d of 80.4 ± 20.7 and 96.3 ± 20.3 pM, respectively (Fig. 2).

Experiment 2

The number of Fos-ir cells in ventrolateral SCN was very low in animals killed in DD for each CT (Fig. 3), fluctuating between 10–40. The Fos-ir was significantly increased by a light pulse given during subjective night (CT14, CT17, CT20, and CT23) and early subjective day (CT2) (Figs. 3,4). On the other hand, light exposure at CT5, CT9, and CT11 did not affect the Fos-ir. A similar light effect on Fos-ir level was found in intact and pinealectomized animals at CT2 (136 ± 15 and 133 ± 7 Fos-ir cells, respectively), as well as at CT5 (37 ± 5 and 29 ± 4 Fos-ir cells, respectively).

DISCUSSION

In a previous study, we showed that the density of melatonin receptors in rat SCN was regulated daily by a mechanism that was dependent on light/dark cycle, and that both light/dark and dark/light transitions had a clear-cut effect on SCN melatonin receptor density (Gauer et al., 1994a). In this study, we show in animals kept in DD that the light pulse-induced increase in melatonin receptor density only occurs during subjective night, and not

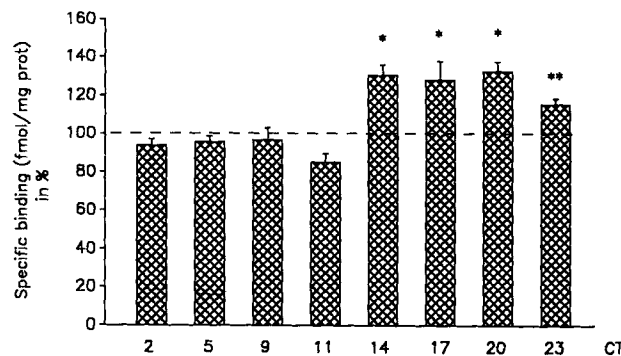


Fig. 1. Relative 2-¹²⁵I-melatonin-specific binding measured in SCN of pinealectomized rats kept in constant darkness for 3 days and sacrificed at eight different circadian times (CT) after a 1-hr-long light pulse. For each CT, the 100% value corresponds to the values of specific binding obtained in the control animals killed in darkness. * $P < 0.01$, ** $P < 0.05$, when compared with respective control values.

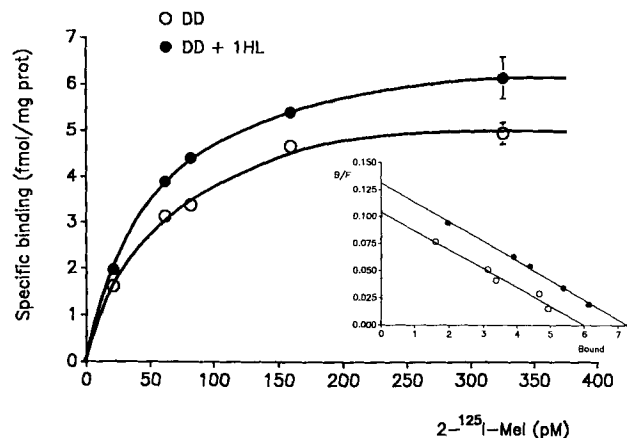


Fig. 2. Saturation curves and Scatchard regression plots of specific 2-¹²⁵I-melatonin binding in SCN of rats pinealectomized for 3 days and subsequently kept in constant darkness. Animals were killed at CT17, after receiving (DD + 1HL) or not (DD) a 1-hr-long light pulse. Each point is mean \pm SEM of 5 animals.

during subjective day (Fig. 1). Therefore, melatonin receptor density responds to light pulses according to a receptor light sensitivity phase, only present during subjective night. The light regulation of melatonin receptors depends, then, on a circadian mechanism.

Similarly, Fos immunoreactivity levels in ventrolateral SCN of pinealectomized rats were increased only when light pulses were applied during subjective night (CT14, CT17, CT20, and CT23), and not when they were administered during subjective day (CT5, CT9, and CT11), as previously reported in intact animals (Rea, 1989; Rusak et al., 1990; Sutin and Kilduff, 1992; Ear-

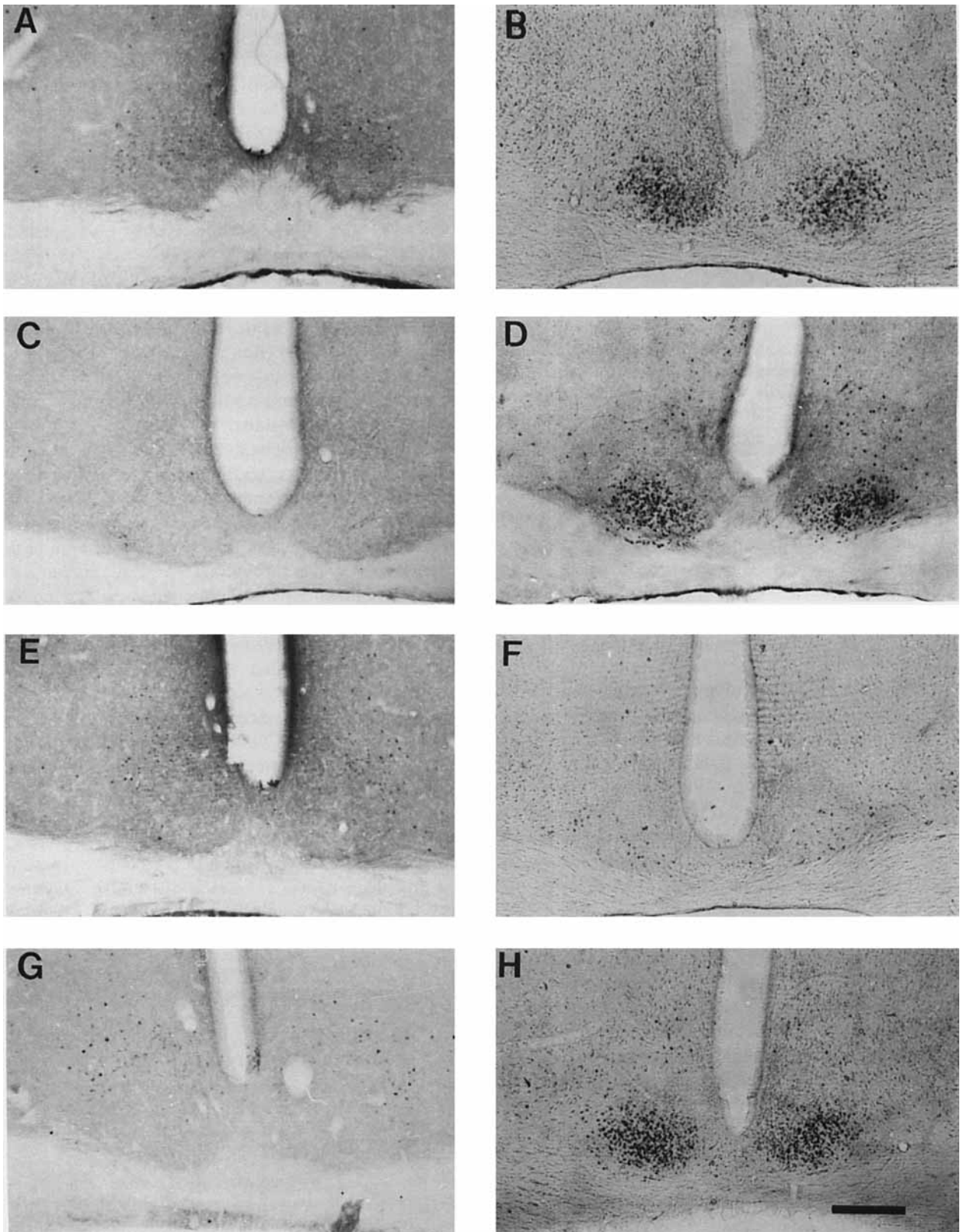


Fig. 3. Endogenous (**left column**) and light-stimulated (**right column**) expression of Fos immunoreactivity in ventrolateral parts of SCN, in pinealectomized rats sacrificed at CT2 (**A, B**), CT5 (**E, F**), and CT20 (**G, H**), and in intact rats sacrificed at CT2 (**C, D**). Bar, 300 μ m.

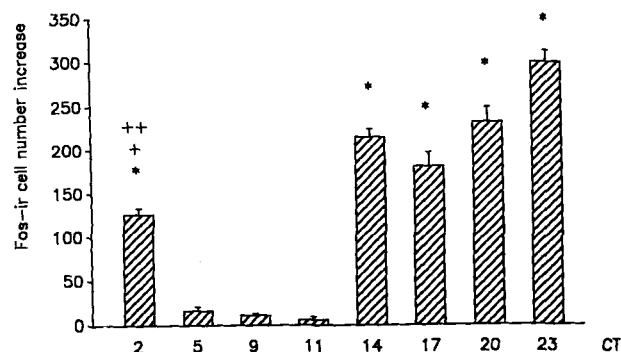


Fig. 4. Increase in number of Fos-immunoreactive (Fos-ir) cells (\pm SEM) in SCN ventrolateral parts of rats pinealectomized for 3 days, subsequently kept in constant darkness and sacrificed at eight different circadian times (CT) after a 1-hr-long light pulse. * $P < 0.01$, compared with values obtained at CT5, CT9, and CT11. + $P < 0.01$, compared with values obtained at CT14, CT20, and CT23. ++ $P < 0.05$, compared with value obtained at CT17.

nest and Oeschowka, 1993; Schwartz et al., 1994). In early subjective day (CT2), however, a less important but clear Fos-ir increase was observed (Figs. 3, 4). This effect (light from CT1–CT2) was not a consequence of pinealectomy, since we found the same results in intact animals, which confirms the results of Schwartz et al. (1994).

The light sensitivity of melatonin receptor density and Fos immunoreactivity restricted to subjective night is also shared by a third phenomenon, the shift of locomotor activity in response to a light pulse (Daan and Pittendrigh, 1976; Summer et al., 1984). Many observations suggest that excitatory amino acids are the neurotransmitters mediating photic effect on the circadian oscillator. For example, the light-induced increases in melatonin receptor density (Gauer et al., 1994b), c-fos gene expression, and activity phase shifts (Abe et al., 1991, 1992; Rea et al., 1993a,b), are blocked by excitatory amino acid receptor antagonists. These observations provide evidence that these different processes are functionally linked. Anatomically, SCN are heterologous structures (Morin, 1994), and different populations of neurons are Fos-light-stimulated according to CTs (Rea, 1992; Chambille et al., 1993). Fos expression has been proposed as a useful marker of neural activation (Sagar et al., 1988). In SCN, Fos induction may thus serve as a general cellular marker for the neural effect of light, whatever the photic-activated circadian function is. Some Fos-activated neuronal populations could be implicated in a cascade of events regulating a given circadian rhythm like melatonin receptor density, while other populations could control other rhythms, such as locomotor activity. This idea is supported by our observation

that during early subjective day Fos was photically elicited, while the density of melatonin receptors was not affected. These Fos-activated cells could then be implicated in the regulation of other rhythms whose candidate could be phase-shift in activity, since a phase advance is observed in the rat at CT2 (Summer et al., 1984). This concept is also supported by the observations that 1) the NMDA receptor antagonist MK 801 totally blocks the light-induced increase in melatonin receptor density (Gauer et al., 1994b), while it only blocks the light-induced c-fos expression in SCN partially (Ebling et al., 1991; Abe et al., 1992; Rea et al., 1993a), and 2) injection of the excitatory amino acid NMDA induces expression of Fos in SCN while it does not cause a phase-shift of the SCN oscillator (Rea et al., 1993b). This possibility does not, however, rule out that Fos activation of some cell populations could result in simultaneous changes in several circadian rhythms.

These results demonstrate the presence of circadian variations in the light regulation of melatonin receptors. The physiological meaning of such a phenomenon is still unknown. However, it could play a major role at dawn and/or dusk, considering that the length of light period is changing every day throughout the year, and that animals are able to adapt daily to these changes in day length. As melatonin is known to entrain circadian locomotor activity (Redman et al., 1983; Cassone, 1990; Kirsch et al., 1993). We believe that a study of melatonin receptor regulation could play a significant role in the search for the endogenous time-keeping mechanism of the circadian pacemaker in SCN. Changes in melatonin receptor density may help to trace events forward along a pacemaker input pathway, ultimately terminating at a component of the oscillatory machinery. Regulation of melatonin receptors might also be considered as a target of the pacemaker. Identification of the factor(s) responsible for this phase-dependent gating mechanism may provide an additional opportunity to trace events backward along one of the pacemaker output pathways.

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