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Chronic exposure to melatonin receptor agonists does not alter their effects on suprachiasmatic nucleus neurons

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Abstract

Previous studies have demonstrated that melatonin and a novel melatonin receptor agonist, S20098 (*N*-[2-(7-methoxy-1-naphthyl) ethyl] acetamide), regulate neuronal firing activity of photically responsive cells in the suprachiasmatic nucleus in vivo. In the present study, we used several different methods to investigate the effects of chronic daily treatment with melatonin, S20098 (1.0 mg/kg, s.c.) or control vehicle for 14 d on responsiveness of suprachiasmatic nucleus cells to these agonists. Both chronic and acute application of drugs were carried out during the day–night transition period. We confirmed that suprachiasmatic nucleus cells from control animals were most sensitive at this circadian phase. Chronic drug treatments did not alter sensitivity of photically responsive suprachiasmatic nucleus cells to S20098 or melatonin given intraperitoneally (i.p.) or iontophoretically in vivo. Suprachiasmatic nucleus cells studied in brain slice preparations also responded similarly to micropressure ejections of melatonin receptor agonists regardless of drug pretreatment. These results indicate that chronic melatonin receptor agonist pretreatment does not result in desensitization of suprachiasmatic nucleus neuronal responses to these agonists during the daily phase of maximum melatonin sensitivity. © 1998 Elsevier Science B.V.

Keywords: Melatonin; Melatonin receptor agonist; Chronic pretreatment; Suprachiasmatic nucleus; Brain slices; Light; Photic response; Entrainment

1. Introduction

The suprachiasmatic nucleus of the hypothalamus functions as the dominant pacemaker for behavioral and physiological circadian rhythms in mammals (for reviews, see Rusak and Zucker, 1979; Moore, 1983). This pacemaker is sensitive to the entraining (synchronizing) effects of daily light exposure, and individual suprachiasmatic nucleus cells in nocturnal rodents respond to retinal illumination, typically with increases in firing rates (Meijer et al., 1986; Ying and Rusak, 1994; Ying et al., 1996; Ying and Rusak, 1997).

The suprachiasmatic nucleus pacemaker regulates the prominent circadian rhythm of melatonin production in the mammalian pineal gland (Klein and Weller, 1970; Moore, 1996). Exogenous melatonin has several effects on suprachiasmatic nucleus function: altering suprachiasmatic nucleus cell responses to light in vivo (Ying et al., 1996), phase-shifting suprachiasmatic nucleus cell firing-rate rhythms in vitro (McArthur et al., 1991, 1997; Margraf and Lynch, 1993), and entraining suprachiasmatic nucleus-driven activity rhythms (Redman et al., 1983; Cassone et al., 1986). The entraining and phase-shifting effects of melatonin are largely restricted to the interval near the day–night transition, with a secondary window of sensitivity near dawn. These circadian effects are likely mediated by high affinity melatonin receptors which have been demonstrated in the suprachiasmatic nucleus of several mammals including Syrian hamsters (Duncan et al., 1988; Williams et al., 1989; Pickering and Niles, 1990; Maywood et al., 1995; Reppert and Weaver, 1995), although melatonin may also act on other targets.

Both pineal melatonin levels and melatonin binding density in the suprachiasmatic nucleus exhibit circadian variations (Laitinen et al., 1989), suggesting the possibility that melatonin regulates its own receptor availability (homologous regulation). However, it remains unclear whether endogenous or exogenous melatonin regulates melatonin receptor status in the suprachiasmatic nucleus, since the

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results from 2-[^{125}I]iodomelatonin binding studies are inconsistent (Laitinen et al., 1992; Gauer et al., 1993; for review, see Gillette and McArthur, 1996). In addition, the functional consequences of chronic melatonin receptor agonist treatment have received little attention, and it is not known whether such treatment alters subsequent sensitivity of suprachiasmatic nucleus cells, including photically responsive cells, to melatonin.

A naphthalenic analog of melatonin, *N*-[2-(7-methoxy-1-naphthyl) ethyl] acetamide (S20098), has been shown to have a very high affinity for 2-[^{125}I]iodomelatonin binding sites (Yous et al., 1992; Bonnefond et al., 1993; Dubocovich et al., 1993). The binding is selective, since S20098 exhibits very low affinities (IC_{50} values $> 10^{-6}$ M) for many other types of receptors ($n = 44$, unpublished observations). S20098 functions as a melatonin receptor agonist in that it mimics the effects of melatonin on several systems (Armstrong et al., 1993; Redman and Guardiola-Lemaître, 1993; Tobler et al., 1994; Redman et al., 1995; Martinet et al., 1996; Ying et al., 1996; Van Reeth et al., 1997).

There is increasing interest in the clinical applications of melatonin receptor agonists as therapeutic agents in humans (for review, see Brzezinski, 1997). The phase-shifting action of melatonin receptor agonists has been applied to treat several circadian rhythm-related disorders, such as jet lag, maladaptation to night shift work, and delayed or advanced sleep phase syndrome (Cajochen et al., 1996; Lewy et al., 1996; for review, see Lewy and Sack, 1996). If melatonin or its analogs are to be used therapeutically in humans, the effects of chronic exposure to them on melatonin receptors and on responsiveness of melatonin target cells to these agonists need to be assessed. We have used several approaches to test for changes in sensitivity of suprachiasmatic nucleus cells recorded in vivo and in vitro to melatonin receptor agonists following chronic treatment of Syrian hamsters with these agonists.

2. Materials and methods

2.1. Pretreatment

Male Syrian hamsters (Charles River, St. Constant, Qué.) weighing 100–145 g were kept in a photoperiod with 14 h of light daily beginning at 05:00 for at least two weeks before being used. For chronic treatment, S20098 (IRIS, France) or melatonin (Sigma) was injected subcutaneously daily for 14 d at a dose of 1.0 mg/kg about 2 h before the onset of darkness. This pharmacological dose has been shown to entrain rat free-running activity rhythms when given by subcutaneous injection, and to inhibit suprachiasmatic nucleus metabolic activity around the day–night transition period (Cassone et al., 1987; Redman and Guardiola-Lemaître, 1993; Redman et al., 1995). Drugs were dissolved in 0.9% NaCl containing 2% DMSO and 1% hydroethylcellulose. Vehicle injections at the same concentration were given to control animals.

2.2. In vivo studies

Following pretreatment of animals for 14 d, electrophysiological experiments were conducted to test suprachiasmatic nucleus cells for their sensitivity to the melatonin receptor agonists. The last chronic injection was given about 20 h before these acute tests. In vivo suprachiasmatic nucleus cells were recorded as previously described (Ying and Rusak, 1994). Briefly, the pretreated hamsters were anesthetized with 20% urethane (2 g/kg, i.p.) at 09:00–10:30, and given subcutaneous injections of Robinul (3-hydroxy-1,1-dimethylpyrrolidinium bromide, α-cyclopentylmandelate, A.H Robins; 0.2%, 0.1-0.2 ml/animal) to reduce respiratory tract congestion during anesthesia. Additional doses of the drugs were given as required. Hamsters were mounted in a stereotaxic apparatus and body temperature was monitored and maintained at 37°C with a thermostatically controlled heating pad throughout the experiment. A hole was drilled in the skull overlying the suprachiasmatic nucleus with the aid of a magnifier. Special care was taken to avoid bleeding caused by damage to the superior sagittal sinus. The eyelids on the side contralateral to the recording site were retracted with sutures and the eye was covered with mineral oil to prevent dehydration after the pupils were dilated with a topical application of 1% atropine sulfate. Glass micropipette electrodes were aimed at the suprachiasmatic nucleus using stereotaxic coordinates (0.2–0.6 mm anterior to bregma, 0.2–0.3 mm lateral to the midline, and 7.6–7.9 mm ventral to the cortical surface), with the upper incisor bar 2 mm below the interaural line. The hamster was maintained in a darkened room except during light pulses or while repositioning the electrode.

In experiments in which drugs were administered i.p., firing activity of suprachiasmatic nucleus cells was recorded with a single glass pipette filled with fast green (Sigma) at a subsaturated concentration in 2 M NaCl. For i.p. injections, melatonin and S20098 were dissolved in 100% DMSO and diluted with normal saline to a final drug concentration of 0.1 mg/ml and a vehicle concentration of 2 or 20%, respectively. In some experiments, multibarrel glass micropipettes were used for recordings and drugs were ejected iontophoretically using a Neurophore BH-2 (Medical Systems, NY), as described previously (Ying et al., 1993, 1996).

Drug solutions were prepared fresh daily. For iontophoresis, melatonin concentration and pH were 5 mM and 4.0–5.0. The amount of melatonin released from a barrel containing 10.0 mM of the drug does not exceed pM concentrations as measured by radioimmunoassay (Stehle et al., 1989). Vehicle solutions containing DMSO at similar concentrations were used as controls for nonspecific effects.

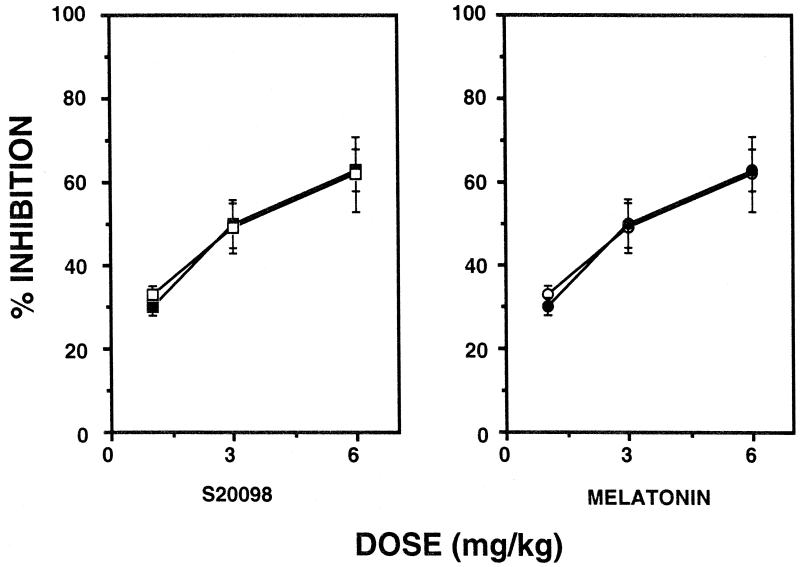

Fig. 1. Dose–response curves showing similar suppression of firing rates of photically responsive suprachiasmatic nucleus cells in vivo by acute injection of 1–6 mg/kg (i.p.) S20098 (left panel) or melatonin (right panel). The hamsters had previously been pretreated chronically with 1.0 mg/kg, s.c. S20098 (left panel: ■), melatonin (right panel: ●) or vehicle (□, ○) for 14 d. Spontaneous firing activity of light-responsive suprachiasmatic nucleus cells was recorded in urethane-anesthetized animals. Each point represents the mean ± S.E.M. of 3–12 cells.

Single-unit extracellular recordings and acute drug tests were carried out during the projected late L and early D phase (16:00–20:00 h, equivalent to Zeitgeber Time (ZT) 11–15, where ZT 0 is light onset). Photically responsive cells and recording sites in the suprachiasmatic nucleus were identified as described previously for studies of neurons in the intergeniculate leaflet and suprachiasmatic nucleus (Ying et al., 1993, 1996; Ying and Rusak, 1994).

2.3. In vitro studies

After being pretreated with melatonin receptor agonists for 14 d, a hamster was anesthetized with halothane, decapitated at approximately 09:00 h, and the brain was rapidly removed. Brain slices (400–450 μm thick) containing the suprachiasmatic nucleus were cut at room temperature with a tissue chopper or a Vibroslice (model VSLM1) in a specimen bath containing artificial cerebrospinal fluid (ACSF) (in mM, NaCl 124, $NaHCO_3$ 25, glucose 10, KCl 3.4, $CaCl_2$ 2.5, $MgSO_4$ 1.0, KH_2PO_4 1.2) gassed with O_2/CO_2 at pH = 7.4. Slices were then incubated in a recording chamber containing ACSF saturated with 95% O_2/5% CO_2 at 35 ± 0.5°C and at a flow rate of 30–35 ml/h.

Slices were allowed to acclimate for at least 1 h before recording. Extracellular recordings of suprachiasmatic nucleus cells and pressure ejection of drugs were performed using single- or multi-barrel glass micropipette electrodes and a Neurophore BH-2 (Medical Systems, NY). To ensure a good ratio of signal to noise and consistent drug release from the micropipettes, the tips of the glass electrodes were carefully broken back to appropriate diameters, and ejection pressures were pretested while inspecting the tip under a microscope.

For pressure ejection, drugs were initially dissolved in 100% DMSO and then diluted with 0.9% NaCl. The final drug concentration was 1.0 mM plus 0.2% DMSO for melatonin and 1.0 mM plus 2% DMSO for S20098. Drugs were pressure-ejected onto suprachiasmatic nucleus cells at pressures of 0.2–6.0 pounds/inch^2 (psi). However, lower

Table 1
Responses of SCN cells in vivo to melatonin receptor agonists

Melatonin effects				
response	pretreatment			
	vehicle		melatonin	
Decrease	14	(67%)	20	(69%)
Increase	1	(5%)	2	(7%)
No change	6	(28%)	7	(24%)
Total	21	(100%)	29	(100%)
S20098 effects				
response	pretreatment			
	vehicle		S20098	
Decrease	21	(75%)	23	(72%)
Increase	3	(11%)	2	(6%)
No change	4	(14%)	7	(22%)
Total	28	(100%)	32	(100%)

Hamsters were kept under a L–D 14:10 cycle and pretreated with melatonin, S20098 (1.0 mg/kg, s.c.) or vehicle daily about 2 h before the onset of darkness for 14 d. Hamsters were anesthetized with urethane and single-unit recordings were made from photically responsive SCN cells. Responses of these cells to i.p. injections of melatonin or S20098 were assessed. A change of at least 20% from pre-drug firing rate was considered a drug effect. Values shown are the number and percentage of cells tested with melatonin or S20098 which showed the response indicated.

pressures (0.5–3.0 psi) were employed routinely to avoid physical effects that can deform the shape of action potentials (Ying and Rusak, unpublished data). Action potential waveforms were monitored throughout drug ejections, and only data from cells showing stable waveforms were retained for analysis. The same vehicle was ejected as a control procedure alternating with drug ejections. Tests of sensitivity of suprachiasmatic nucleus cells to melatonin

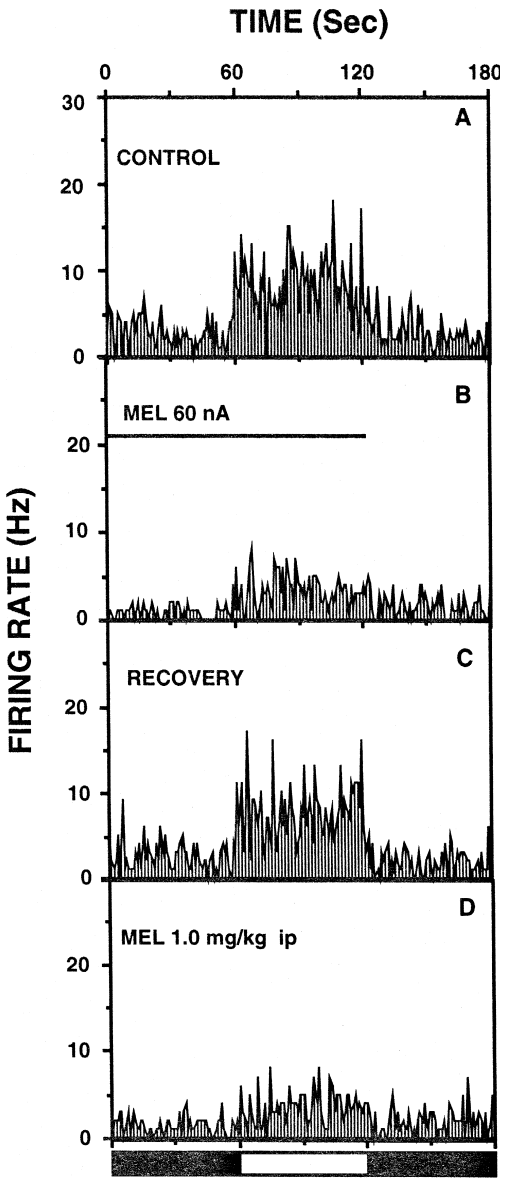

Fig. 2. Integrated firing rate histogram illustrating similar suppression of photic responses of suprachiasmatic nucleus cells in vivo by melatonin applied locally (by iontophoresis) or systemically (i.p. injection) in a melatonin-pretreated hamster. The white bar at the bottom indicates the duration and timing of retinal light exposure, while the horizontal line in B indicates the timing of melatonin iontophoresis. See legend to Fig. 1 for other details. (A) Baseline photic response. (B) Iontophoresis of melatonin at a current of 60 nA for 2 min decreased the spontaneous firing rate and the photic response. (C) Recovery from suppression after 10 min. (D) A similar suppressive effect resulting from i.p. injection of melatonin.

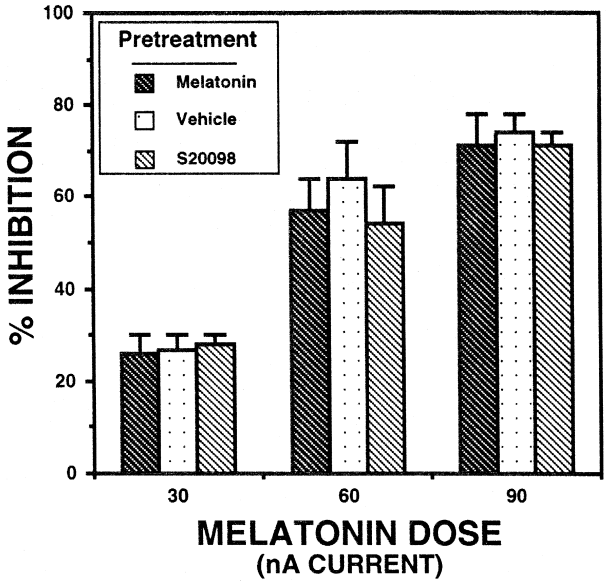

Fig. 3. Comparison of the effects of pretreatment with melatonin, S20098 or vehicle on responses to melatonin of hamster suprachiasmatic nucleus cells recorded in vivo. Recordings were done as described in Fig. 1, but melatonin was applied to suprachiasmatic nucleus cells by iontophoresis only. Each point represents the mean ± S.E.M. of 3–6 cells.

receptor agonists were carried out during ZT 11–15, which corresponds to the previously reported time of peak sensitivity to melatonin (Gillette and McArthur, 1996), and results were compared to data collected during ZT 5–7.

2.4. Data acquisition and analyses

Data acquisition, light exposures and iontophoretic applications of drugs for in vivo studies were controlled by a computer program (Pulsecount) written by A. Hurshman (Ying et al., 1993, 1996). In in vitro experiments, extracellular recordings were done similarly, but discriminated signals were fed into a frequency counter, and then plotted as an integrated firing-rate histogram on a pen recorder (Linear, USA, Model 500). Agonist effects were defined as changes relative to predrug firing rates of at least 20% at some dose of a drug. In order to compare drug effects, agonist potencies were also expressed as ED_{50} values: the doses (= ejection pressures) required to produce half-maximal effects, as described previously (Ying et al., 1996). Statistical analyses of drug effects included unpaired *t*-tests, contingency analysis, one-way ANOVA and regression analysis (Systat 5.2.1, Systat, IL).

3. Results

3.1. In vivo experiments

A total of 146 photically responsive suprachiasmatic nucleus cells were recorded in vivo and tested for their

sensitivity to melatonin receptor agonists given i.p. or iontophoretically after hamsters were pretreated with these agonists for 14 d. Cells showing apparent responses (> 20% change in firing rates) to vehicle alone were excluded from the analysis. Cells were identified as being in or near the suprachiasmatic nucleus based on histological reconstructions of recording sites.

Systemic injections (i.p.) of melatonin effectively decreased spontaneous firing rates of the majority of suprachiasmatic nucleus cells in both melatonin-pretreated and vehicle-pretreated hamsters (Fig. 1); only a few cells were activated by the drug (Table 1). These data were analyzed by contingency analysis which showed that there was no significant difference between melatonin-pretreated and vehicle-pretreated animals ($X^2 = 0.0988$, $P \gg 0.05$). The average suppression of firing activity was $38.9 \pm 8.8\%$ (mean $\pm$ S.E.M.) in animals pretreated with vehicle and $36.3 \pm 5\%$ in those pretreated with melatonin. There was no significant difference between pretreatment with the drug and vehicle, as revealed by an unpaired t-test ($t = 0.337$, $P > 0.05$).

Similarly, melatonin pretreatment did not attenuate effects of the hormone on photic responses of suprachiasmatic nucleus cells. Typical responses of a light-sensitive suprachiasmatic nucleus cell to i.p. melatonin injection are shown in Fig. 2.

Systemic injections of S20098 also decreased spontaneous firing rates in the majority of suprachiasmatic nucleus cells (Table 1), and caused an average suppression of $46.7 \pm 8.8\%$ in vehicle-pretreated animals and $44.4 \pm 5.1\%$ in S20098-pretreated animals (Fig. 1). The sensitivity of suprachiasmatic nucleus cells to S20098 administration was not significantly affected by pretreatment with this agonist, as compared to vehicle-treated animals (contingency analysis, $X^2 = 1.309$, $P \gg 0.05$, and t-test, $t = 0.303$, $P > 0.05$). Also, S20098 pretreatment did not alter

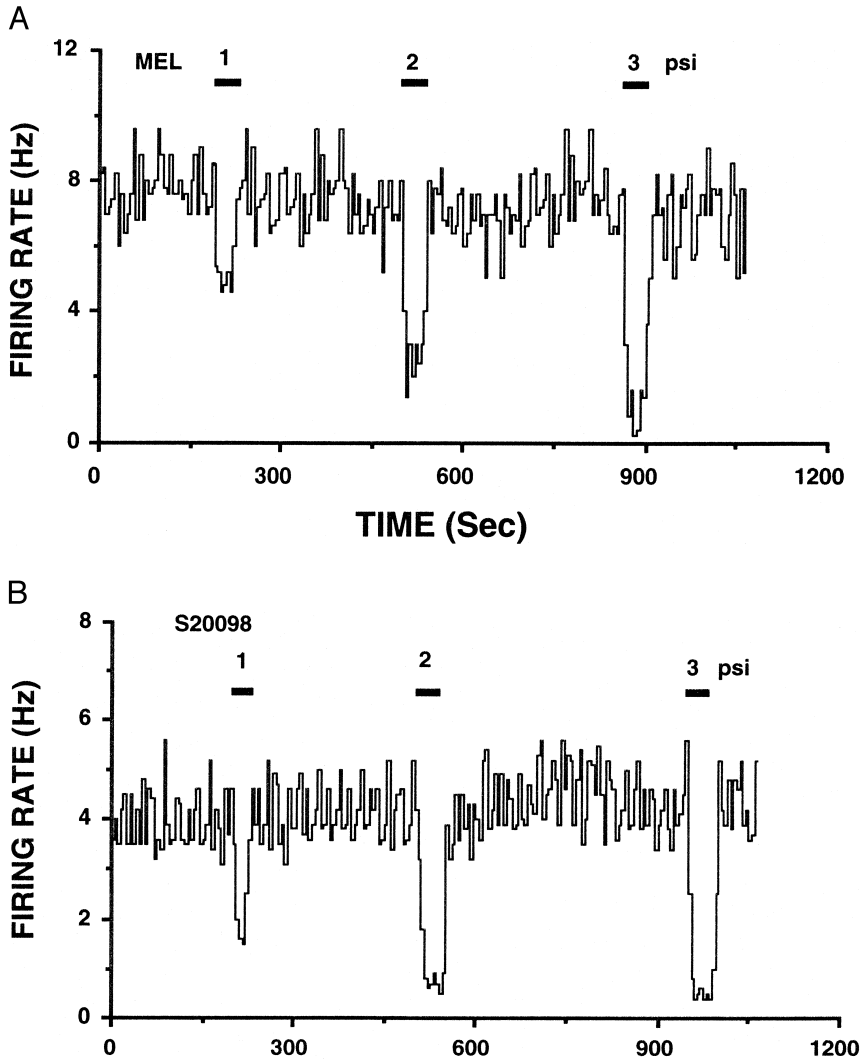

Fig. 4. Responses of suprachiasmatic nucleus cells to melatonin receptor agonists in brain slices. Drugs were applied to suprachiasmatic nucleus cells by micropressure ejection. Animals had been pretreated with melatonin (A) or with S20098 (B) as described in the legend to Fig. 1. The horizontal bar and number above the record indicate the timing and ejection pressure (in psi) of drug applications.

Table 2
Responses of SCN cells in vitro to melatonin receptor agonists

Melatonin effects				
response	pretreatment			
	vehicle		melatonin	
Decrease	26	(59%)	23	(52%)
Increase	6	(14%)	6	(14%)
No change	12	(27%)	15	(34%)
Total	44	(100%)	44	(100%)
S20098 effects				
response	pretreatment			
	vehicle		S20098	
Decrease	23	(58%)	39	(59%)
Increase	5	(12%)	7	(11%)
No change	12	(30%)	20	(30%)
Total	40	(100%)	66	(100%)

Hamsters were kept under a L–D 14:10 cycle and pretreated with melatonin, S20098 (1.0 mg/kg, s.c.) or vehicle daily about 2 h before the onset of darkness for 14 d. Brain slices were prepared and extracellular recordings made from SCN cells during micropressure ejection of melatonin receptor agonists. A change of at least 20% from pre-drug firing rate was considered a drug effect. Values shown are the number and percentage of cells tested with melatonin or S20098 which showed the response indicated.

the drug's effects on photic responses of suprachiasmatic nucleus cells (data not shown).

A final comparison between responses in melatonin-, S20098-, and vehicle-pretreated animals was performed by one-way ANOVA, which yielded $F_{3,121} = 0.097$, $P = 0.962$. Therefore, prior exposure to either agonist did not alter subsequent sensitivity of suprachiasmatic nucleus cells in vivo to the agonists given by i.p. injections.

To assess whether indirect effects of systemic drug injections might have obscured changes in direct sensitivity of suprachiasmatic nucleus cells, we also applied melatonin directly to single suprachiasmatic nucleus cells by microiontophoresis through a multibarrel micropipette. In a subgroup of suprachiasmatic nucleus cells tested, iontophoretic application of melatonin dose-dependently depressed firing rates of suprachiasmatic nucleus cells in animals pretreated with melatonin, S20098 or vehicle (Fig. 3). Melatonin was similarly effective on single cells whether given by local or systemic administration (Fig. 2). Inhibitory responses to melatonin were very similar in all groups and the average suppressions (mean ± S.E.M.) at an ejection current of 90 nA were 74 ± 4%, 71 ± 7%, and 71 ± 3% in vehicle-, melatonin- and S20098-pretreated animals, respectively. Statistical analysis showed that there were no significant differences in melatonin effects between vehicle-, melatonin- and S20098-pretreated animals ($F_{2,41} = 0.369$, $P = 0.694$).

Cells that were tested with both melatonin and S20098 were similarly responsive to both drugs, as reported previously (Ying et al., 1996). This finding is consistent with the interpretation that S20098 acts at melatonin receptors (Yous et al., 1992).

3.2. In vitro experiments

Since most other studies of melatonin receptor regulation have used in vitro preparations, we examined the effects of melatonin receptor agonists on suprachiasmatic nucleus cells in hypothalamic slices prepared from pretreated animals. Micropressure ejection of melatonin effectively decreased firing rates of most suprachiasmatic nucleus cells (Fig. 4A), although a small number of cells

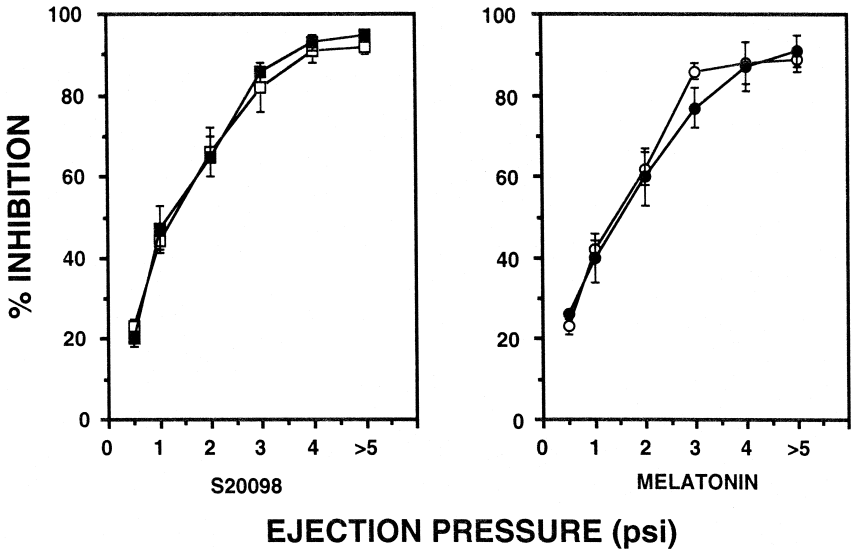

Fig. 5. Dose–response curves demonstrating similar suppression of firing rates of suprachiasmatic nucleus cells in response to micropressure ejection of S20098 (left panel) and melatonin (right panel) in a brain slice preparation. The hamsters had previously been pretreated with 1.0 mg/kg, s.c. S20098 (●), melatonin (■) or vehicle (□, ○) for 14 d. before acute tests. Each point represents the mean ± S.E.M. of 3–10 cells.

were activated by the drug (see Table 2). Again, there was no significant difference in melatonin effects between melatonin- and vehicle-pretreated groups ($X^2 = 0.094$, $P > 0.05$). This suppression was strongly dose (pressure)-dependent (Fig. 5), and yielded ED_{50} values of 1.63 and 1.66 psi, with maximal suppressions at 3.0 psi of $80 \pm 4\%$, and $77 \pm 3\%$ for vehicle- and melatonin-pretreated animals, respectively. The sensitivity of suprachiasmatic nucleus cells to this agonist was not significantly altered by melatonin pretreatment (unpaired t-test, $t = 0.312$, $P > 0.05$), as compared to the vehicle-pretreated group.

Pressure ejection of S20098 also effectively depressed firing rates of suprachiasmatic nucleus cells recorded in vehicle- and S20098-pretreated animals, as seen in Table 2, Fig. 4B. The suppression was also dose-dependent (Fig. 5), with ED_{50} values of 1.50 and 1.44 psi and maximal suppressions at 3.0 psi of $82 \pm 4\%$ and $86 \pm 5\%$ for vehicle- and S20098-pretreated animals, respectively. Statistical analyses revealed that there was no significant difference in the effects of S20098 on suprachiasmatic nucleus cells from drug- and vehicle-pretreated animals of S20098 (contingency analysis, $X^2 = 0.0904$, $P > 0.05$, and unpaired t-test, $t = 0.196$, $P > 0.05$).

Finally, one-way ANOVA was performed to compare differences between effects produced by agonist- and vehicle-pretreatment. This analysis revealed that there were no significant differences between melatonin-, S20098- and vehicle-pretreated animals ($F_{3,190} = 0.073$, $P = 0.974$).

We also assessed whether previously reported temporal changes in sensitivity to melatonin were apparent in our system by comparing the responsiveness of suprachiasmatic nucleus cells from vehicle-treated animals to melatonin during two different time windows. More cells responded to the drug with suppression during ZT 11–15 (spanning the projected L–D transition) than during ZT 5–7, when most cells were unresponsive (t-test, $t = 3.618$, $P = 0.001$; see Fig. 6).

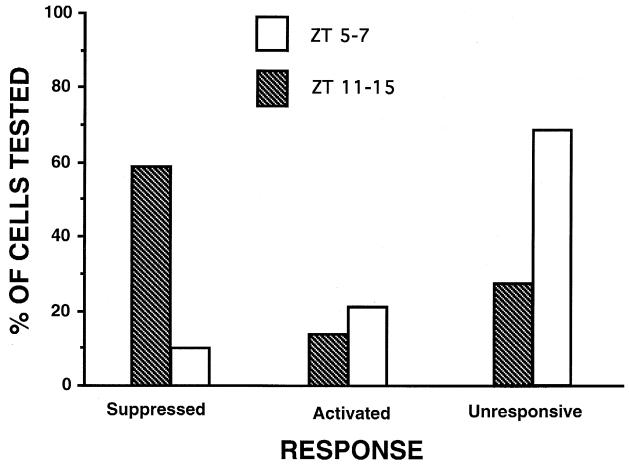

Fig. 6. Sensitivity of hamster suprachiasmatic nucleus cells in vitro to melatonin, assessed during two time periods. Brain slices were prepared from vehicle-treated hamsters which were maintained under a L–D 14:10 cycle with lights on at 05:00 h until slice preparation. Melatonin was applied to suprachiasmatic nucleus cells during 10:00–12:00 h (open bar, ZT 5–7) or 16:00–20:00 h (hatched bar; ZT 11–15).

4. Discussion

Several approaches to studying receptor sensitivity yielded the consistent finding that chronic administration (14 d) of melatonin receptor agonists at a pharmacological dose did not result in a loss of sensitivity of suprachiasmatic nucleus cells to these drugs. Assuming that these effects are mediated by melatonin receptors in the Syrian hamster suprachiasmatic nucleus (Pickering and Niles, 1990; Maywood et al., 1995), this finding is consistent with the observation that daily melatonin injections (1.0 mg/kg, s.c.) do not affect density or affinity of melatonin receptor binding in the suprachiasmatic nucleus (Laitinen et al., 1992). Our results are also consistent with the observation that entraining effects of melatonin and S20098 on circadian activity rhythms persist during daily injections of either agonist at a pharmacological dose (1.0 mg/kg, s.c.) for over 16 d (Redman and Guardiola-Lemaître, 1993; Redman et al., 1995) and for more than 50 d in other studies (Redman et al., 1983; Marumoto et al., 1996).

Homologous (agonist) regulation of G protein-coupled receptors has been reported for other receptors (Sibley and Lefkowitz, 1985; Hausdorff et al., 1990). However, alteration of endogenous melatonin levels has not consistently produced the consequences associated with receptor down- or up-regulation. For instance, acute light exposure for 12 h, pinealectomy, or superior cervical ganglionectomy does not affect affinity or density of binding to melatonin receptors in the rat suprachiasmatic nucleus and other brain areas (Laitinen et al., 1992). In contrast, pinealectomy or 72 h of constant light can alter the density of receptor binding in the rat suprachiasmatic nucleus and pars tuberalis, and the increased receptor density can be reversed by a single exogenous melatonin treatment (50 μg/rat) (Gauer et al., 1993). Similarly, constant light or pinealectomy can alter patterns of suprachiasmatic nucleus cell sensitivity to melatonin in hamsters (Rusak and Yu, 1993; Yu et al., 1993). It is possible that treatments which alter physiological levels and patterns of melatonin cause transient or circadian-phase dependent changes in receptor numbers that are not evident following long-term treatments with pharmacological doses.

In our experiments, over 20 h elapsed between the last chronic injection and acute tests of sensitivity to melatonin receptor agonists. Receptor turnover after exposure to the agonist could be an important issue: there may have been sufficient time for receptor turnover before acute tests, since melatonin has a half-life of about 35 min after subcutaneous injection in rats (Cassone et al., 1986). This

feature was not assessed since the studies were not aimed at detecting transient down- or up-regulation of receptors which might have intervened between the last pretreatment and the recording session. Instead, the goal was to assess whether daily treatment with melatonin receptor agonists in a pattern appropriate for therapeutic interventions aimed at suprachiasmatic nucleus function would alter sensitivity to these drugs by the next scheduled treatment time. The present results indicate that chronic pretreatment does not desensitize suprachiasmatic nucleus cells to these melatonin receptor agonists given at the phase when melatonin has its most potent phase-shifting effects (Redman et al., 1983; Stehle et al., 1989; McArthur et al., 1991, 1997; Margraf and Lynch, 1993; Marumoto et al., 1996).

A relative lack of desensitization was also reported in studies of the ovine pars tuberalis (Hazlerigg et al., 1993). Following pretreatment with melatonin (100 nM) for 16 h, a high (1.0 μM) concentration of melatonin still potently inhibited forskolin-stimulated cAMP accumulation, indicating the persistence of sensitivity, although the IC_{50} was increased for lower concentrations (pM–nM) of the agonist.

The lack of desensitization we observed cannot be attributed to melatonin acting on other brain areas during acute tests to indirectly alter suprachiasmatic nucleus cell activity, since similar results were obtained with systemic and local drug administrations. In particular, some cells which were depressed by melatonin given by i.p. injection were also shown to be responsive to this drug when applied iontophoretically. Similar results were also obtained using micropressure ejection of the agonists onto neurons recorded in suprachiasmatic nucleus slice preparations.

One possible explanation for the lack of desensitization is that spare melatonin receptors are available in the suprachiasmatic nucleus, and occupancy of all melatonin receptor sites is not necessary for a maximal neuronal response (Hazlerigg et al., 1993). This hypothesis, however, can be challenged by the fact that the high affinity receptor in the hamster hypothalamus is saturable by the presence of pharmacological levels (nM) of the agonist (Duncan et al., 1988; Laitinen et al., 1989; Williams et al., 1989; Pickering and Niles, 1990; Maywood et al., 1995).

Suprachiasmatic nucleus cell sensitivity to melatonin appears to be regulated more strongly by an intrinsic circadian rhythm than by the history of agonist availability. For example, even in hypothalamic slices cultured in the absence of melatonin for 1–3 d, suprachiasmatic nucleus cells continue to show a rhythm of sensitivity to melatonin (Margraf and Lynch, 1993; Yu et al., 1993; McArthur et al., 1997). In our in vitro experiments suprachiasmatic nucleus cells from hamsters pretreated with vehicle showed reduced sensitivity to melatonin given during ZT 5–7, and high sensitivity during ZT 11–15. Although it is possible that homologous regulation of melatonin receptor availability occurs on some time scale, these results reinforce the conclusion that responsiveness of the suprachiasmatic nucleus to melatonin is regulated strongly by the circadian clock (Stehle et al., 1989; Margraf and Lynch, 1993; Gillette and McArthur, 1996; McArthur et al., 1997).

In conclusion, our results imply that chronic use of melatonin receptor agonists for therapeutic purposes aimed at suprachiasmatic nucleus function may not be complicated by the development of desensitization during the phase of peak melatonin sensitivity, over the course of at least two weeks. It remains to be assessed whether continued treatment beyond this interval would result in altered cellular sensitivity to agonists.

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