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Physiological regulation of melatonin receptors in rat suprachiasmatic nuclei: diurnal rhythmicity and effects of stress

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Summary

A marked diurnal variation in high-affinity binding of 2-[^{125}I]iodomelatonin ([^{125}I]MEL) in rat brain sections containing the suprachiasmatic nuclei (SCN) was observed. Binding was highest late in the light phase and lowest during darkness, in inverse correlation to the serum melatonin rhythm. Interestingly, only high-affinity sites were present during most of the light phase while both high- and low-affinity sites were detected just before and during darkness. Guanosine triphosphate (GTP) in combination with sodium converted all high affinity sites to a low affinity state suggesting that the two sites observed during darkness represent the two states of the melatonin receptor. Acute swim-stress caused a significant elevation of serum melatonin, together with a decrease in the density of [^{125}I]MEL binding in the SCN. The inverse relationship between circulating melatonin levels and binding, under two different physiological conditions, indicates that this hormone is involved in regulating its own receptors in the SCN.

Introduction

The suprachiasmatic nuclei (SCN) are thought to be the principal pacemakers that generate mammalian circadian rhythmicity in a variety of biological activities including the daily production of the pineal hormone, melatonin (Redman et al., 1983; Takahashi and Zatz, 1982). It has been suggested that melatonin, secreted by the pineal gland, influences the regulation of circadian cyclicity by the SCN. For example, the free running circadian rhythm in rat locomotor activity can be entrained by melatonin injections (Cassone et al., 1986a), while this effect is prevented by ablation of the SCN (Cassone et al., 1986b). These studies suggest that the SCN contain target sites for melatonin. This point of view is further supported by the fact that 2-deoxyglucose uptake in the SCN is inhibited by injection of melatonin (Cassone et al., 1987).

Since the high doses of melatonin used in these studies might have produced pharmacological effects on various CNS systems (Niles and Hashemi, 1990a; Niles and Peace, 1990), the physiological relevance of these findings is questionable. However, with the development of radioiodinated melatonin (2-[^{125}I]iodomelatonin), autoradiographical studies have indicated that this selective melatonin receptor probe binds to discrete regions of the mammalian brain including the SCN (Krause and Dubocovich, 1990). In addition, a low concentration (1 nM) of melatonin, which approaches its physiological levels, was recently found to alter the electrical activity rhythm of the SCN in vitro (McArthur et al., 1991). Therefore, it is quite likely that physiological regulation of SCN activity by melatonin occurs. In order to characterize the high-affinity binding sites for melatonin in the SCN, we have examined the rostro-caudal and diurnal characteristics of 2-[^{125}I]iodomelatonin ([^{125}I]MEL) binding in brain sections containing these nuclei. In addition, the effect of acute stress was examined in studies aimed at correlating changes in endogenous melatonin levels with [^{125}I]MEL binding.

Materials and methods

Animals

Adult male Sprague-Dawley rats (Charles River) were housed in groups of three in a temperature con-

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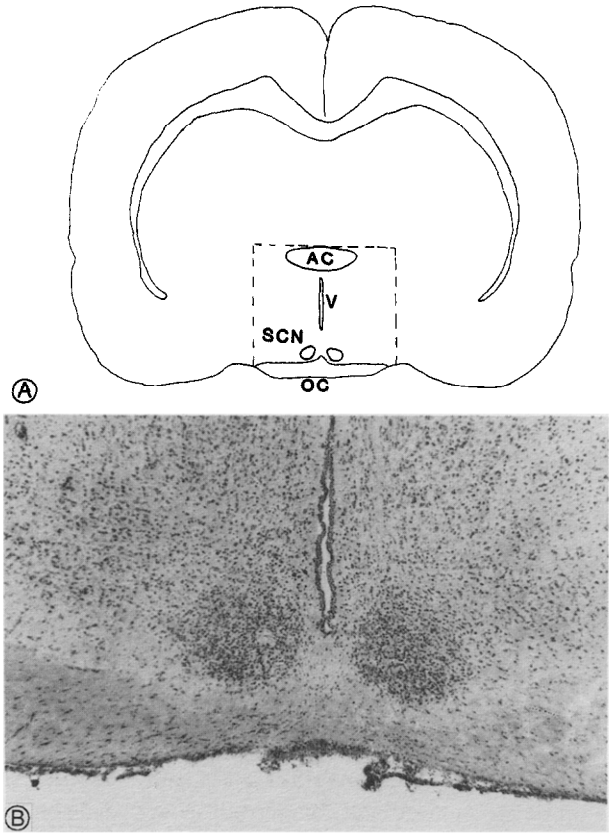


Fig. 1. (A) A diagrammatic representation of the hypothalamic block used for preparing sections containing the suprachiasmatic nuclei. (B) A representative hypothalamic section showing the suprachiasmatic nuclei stained with Toluidine Blue.

trolled environment with lights on at 08 00 h and off at 20 00 h. Food and water were provided ad libitum, and the animals were acclimatized to animal quarters for at least a week before the start of experiments.

Materials

[^{125}I]MEL, specific activity ~ 1200–1400 ci/mmol, was synthesized and purified by HPLC as previously detailed (Niles et al., 1987). Melatonin was purchased from Sigma. All other reagents were obtained from commercial sources.

Tissue preparation

Rats (weighing 250–300 g) were decapitated and brains dissected free of blood vessels and connective tissue, and frozen in cold isopentane (−20°C). A block of hypothalamic tissue containing the suprachiasmatic nuclei (SCN) was cut in a cryostat at −16°C (see Fig. 1). Coronal sections (20 μm thick) were collected, starting from the origin of the optic chiasm to a distance of 900 μm caudally. The tissue sections were placed directly into prechilled test-tubes, and subsequently transferred to centrifuge tubes and homogenized by a hand-held homogenizer (20 strokes), in 10 ml of 50 mM Tris-HCl (pH 7.4 at 4°C). The homogenate was centrifuged at 39000 × g for 10 min and the pellet was washed twice by resuspension in 10 ml of buffer and centrifugation at 39000 × g for 10 min.

Binding assays

Homogenates were incubated at room temperature (20–22°C) for 1 h with 60–70 pM of [^{125}I]MEL in single-point experiments. In saturation experiments, homogenates were incubated with [^{125}I]MEL ranging from about 5 pM to 30 nM. Radioligand concentrations exceeding 5 nM were obtained by isotopic dilution of [^{125}I]MEL as previously reported (Pickering and Niles, 1990). Nonspecific binding was determined in the presence of 1 μM melatonin. Bound radioactivity was separated by filtering under vacuum and filters were transferred into glass tubes and counted in an LKB gamma counter.

Diurnal studies

In diurnal studies of [^{125}I]MEL binding, animals were killed at 02 00 h, 07 30 h, 09 00 h, 12 00 h, 16 00 h, 18 00 h, 19 30 h, 20 00 h and 24 00 h. Brains were dissected, frozen in cold isopentane (−20°C) and stored at −70°C until assayed. During the dark period, animals were killed under red light in order to avoid suppression of melatonin synthesis by light. Trunk blood was collected and serum samples were stored at −20°C until used to measure melatonin levels by a radioreceptor assay (Tenn and Niles, 1991). Analysis of saturation data was carried out by the computer programme BDATA (EMF software) as previously reported (Pickering and Niles, 1990; Ying and Niles, 1991). Data were statistically evaluated by one-way analysis of variance (ANOVA) and Scheffe's test or by unpaired Student's t-test where appropriate.

Stress procedure

The stress study was carried out at 16 00 h since diurnal studies indicated that high-affinity binding was highest at this time (see Figs. 3 and 4). The control group went untreated and was sacrificed as quickly as possible in order to minimize stress. The experimental group swam (one at a time) for 10 min in a plastic container filled with room temperature water (19–21°C), sufficiently deep that they could neither touch the bottom (approximately 22 cm) nor climb out the top. After 10 min the animals were taken out of the water and returned to their housing. Twenty minutes later (i.e. 30 min after swimming onset), the rats were killed by decapitation, trunk blood was collected and brains were dissected and frozen on solid CO_2 and stored at −20°C until use. Blood samples were refrigerated for 1 h, centrifuged and serum was stored at −20°C for future assay. All experimentation and killing procedures were carried out in a separate room from where the animals were housed.

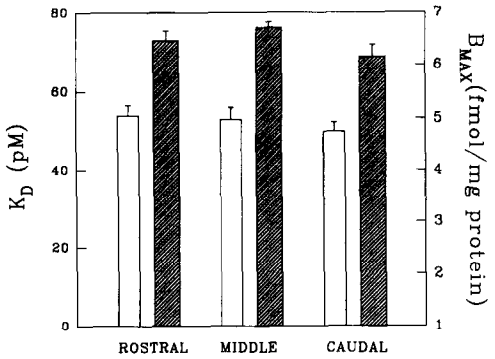


Fig. 2. Saturation binding of [^{125}I]MEL in homogenates prepared from rostro-caudal sections of rat hypothalamus. Sections were pooled for each indicated area which covered a distance of about 300 μM along the rostro-caudal length of the SCN. Means ± SEM of triplicate determinations are presented.

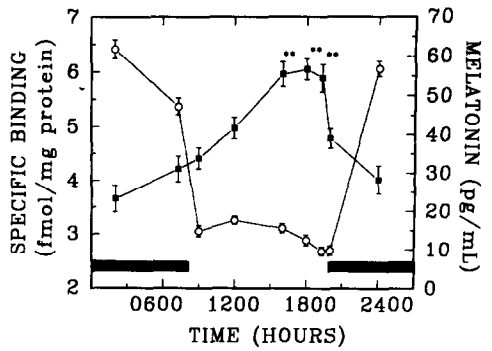


Fig. 3. Diurnal rhythms of serum melatonin (○) and [^{125}I]MEL binding in the rat SCN (■). Means ± SEM of three separate experiments conducted in triplicate are presented. Solid bars indicate period of darkness. $^{**}p < 0.01$ vs all other points (Scheffe's test).

Results

Rostro-caudal binding

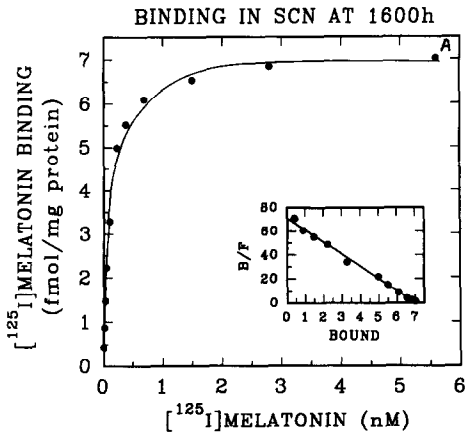
In preliminary experiments, little or no binding of [^{125}I]MEL was detected in hypothalamic sections just anterior to or posterior to the SCN, as compared with sections containing these nuclei. A comparison of binding in sections taken from the rostral, middle and caudal regions of the SCN (approximately 300 μm per region) revealed no significant differences in either binding affinity or density: – rostral region: $K_d = 54 \pm 2$ pM, $B_{max} = 6.5 \pm 0.1$ fmol/mg protein; middle region: $K_d = 53 \pm 2$ pM, $B_{max} = 6.7 \pm 0.1$ fmol/mg protein; caudal region: $K_d = 50 \pm 1$ pM, $B_{max} = 6.2 \pm 0.1$ fmol/mg protein (Fig. 2).

Diurnal binding

Serum melatonin exhibited a marked circadian rhythm with low levels during the light period and a crest in the dark period (ANOVA: $F = 518$; df = 8,18; $p < 0.001$). Specific [^{125}I]MEL binding in the SCN displayed an opposite rhythm to serum melatonin (Fig. 3), with a peak in the latter part of the light phase and a trough after 4–6 hours of darkness (ANOVA: $F = 49$; df = 8,18; $p < 0.001$). Saturation data indicated the presence of a single high-affinity site during most of the light phase, while both high- and low-affinity sites were detected 30 min before lights were turned off and during the dark period (Figs. 4 and 5). The B_{max} for the high-affinity site was significantly higher at 1600 hours than at any other time (Table 1 and Fig. 5). With the appearance of two binding sites, there was a gradual decrease in the density of high-affinity sites during darkness. Conversely, the concentration of low-affinity sites increased significantly during the dark phase and there appeared to be a concomitant decrease in the affinity of these sites but this was not significant (Table 1 and Fig. 5).

Effect of GTP and sodium

On the assumption that the single site detected during the light phase was the high-affinity state of the melatonin receptor, the effect of the affinity modulators, GTP and sodium, were examined on binding at 16 00 h. In single point experiments, incubation with either GTP (1 mM) or sodium chloride (150 mM)



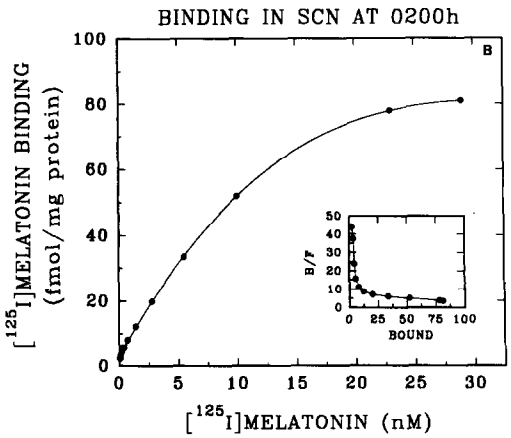


Fig. 4. Saturation binding of [^{125}I]MEL in the SCN at 1600h (A) and 02 00 h (B). The radioligand was used in concentrations of 5 pM – 5.6 nM (Fig. 4A) or 5 pM – 30 nM (Fig. 4B). Insets: Scatchard plots of data. Means of triplicate values from one of 3–4 separate experiments are presented.

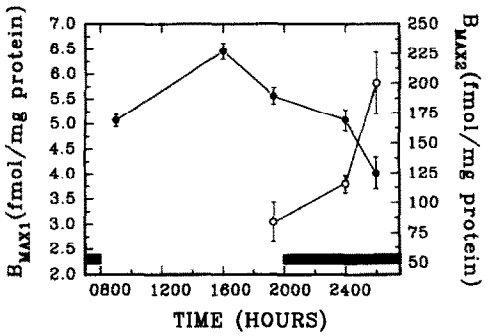


Fig. 5. Diurnal variations in the density of high- and low-affinity binding sites for [^{125}I]MEL. Means ± SEM of 3–4 separate experiments conducted in triplicate are presented. Solid bars indicate period of darkness. High-affinity: B_{max} (●), Low-affinity: B_{max} (○).

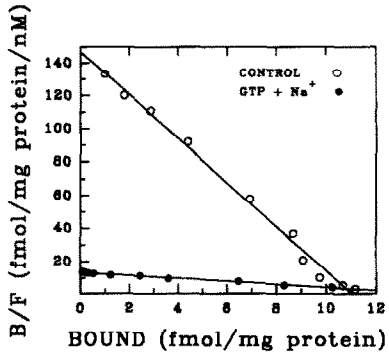


Fig. 6. Scatchard plots of [^{125}I]MEL binding in hypothalamic membranes in the absence (○) and presence (●) of GTP (1 mM) + NaCl (150 mM). Means of triplicate determinations from one representative experiment, which was replicated 3–6 times, are presented. See text for values of binding parameters.

reduced binding by ~40% while a combination of these agents was even more effective and suppressed binding by ~60%. In saturation experiments, incubation with GTP or sodium caused a two-fold decrease in binding affinity but the B_{max} was unchanged. However, a combination of these agents produced an eighteen-fold decrease in affinity and converted all high-affinity sites to a low-affinity state (see Fig. 6). Dissociation constants in the absence and presence of the above modulators were: control: $K_d = 62 \pm 5$ pM, $n = 6$; GTP/Na^+: $K_d = **1120 \pm 1$ pM, $n = 3$ ($**p < 0.001$ vs control, Student's t-test). Corresponding binding site densities were: control: $B_{max} = 8 \pm 1$ fmol/mg protein, $n = 6$; GTP/Na^+: $B_{max} = *18 \pm 4$ fmol/mg protein, $n = 3$ ($*p < 0.01$ vs control, Student's t-test).

Effects of stress

Animals subjected to a forced 10-min swim exhibited a significant ($p < 0.01$, Student's t-test) increase of about 3-fold in serum melatonin levels (Fig. 7). Conversely, [^{125}I]MEL binding was lower in the SCN of stressed animals (Fig. 7), as a result of a significant ($p < 0.01$) decrease in the density of binding sites, but there was no change in affinity (Fig. 8). Dissociation constants and binding site densities were: control: $K_d = 52 \pm 1$ pM, $B_{max} = 6.4 \pm 0.2$ fmol/mg protein, $n = 3$; stress: $K_d = 47 \pm 2$ pM, $B_{max} = **3.3 \pm 0.1$ fmol/mg protein, $n = 3$ ($**p < 0.001$ vs control, Student's t-test).

TABLE 1

DIURNAL CHARACTERISTICS OF [^{125}I]MEL BINDING IN THE SCN

Data are means ± SEM of the number of saturation binding experiments indicated in parentheses.

Time (h)	K_{d_1} (pM)	B_{max_1} (fmol/mg protein)	K_{d_2} (nM)	B_{max_2} (fmol/mg protein)
09 00	37.9 ± 1.3 (3)	5.1 ± 0.1 (3)	–	–
16 00	39.0 ± 0.5 (3)	**6.5 ± 0.2 (3)	–	–
19 30	40.7 ± 5.3 (3)	5.6 ± 0.2 (3)	2.7 ± 0.4 (3)	84.0 ± 16.4 (3)
24 00	39.7 ± 2.9 (3)	5.1 ± 0.2 (3)	3.1 ± 0.3 (3)	115.5 ± 7.4 (3)
02 00	30.1 ± 1.31 (4)	4.0 ± 0.3 (4)	12.43 ± 4.0 (4)	*200.4 ± 25.7 (4)

– = Not detected.
** $p < 0.01$ vs B_{max_1} at all other times; * $p < 0.05$ vs B_{max_2} at 19 30 h.

Discussion

In the present study, the diurnal rhythm of [^{125}I]MEL binding to the high-affinity site in the SCN was found to be inversely correlated with circulating melatonin levels. Single-point binding increased gradually during the light phase and peaked after about 8–10 h of light exposure, during which time serum melatonin levels were depressed. In a departure from this inverse correlation between binding and hormone levels, there was a significant decrease in binding at the point of light-dark transition (20 00 h), although circulating melatonin levels were still low, as shown in Figure 3. More-

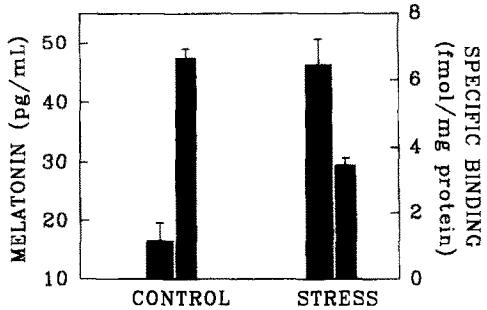


Fig. 7. Effects of swim-stress on serum melatonin levels and [^{125}I]MEL saturation binding in the SCN. Stress caused a significant ($p < 0.01$) increase in melatonin levels (■) and a significant ($p < 0.01$) decrease in the density (□) of [^{125}I]MEL binding sites. Means ± SEM of three separate experiments conducted in triplicate are presented.

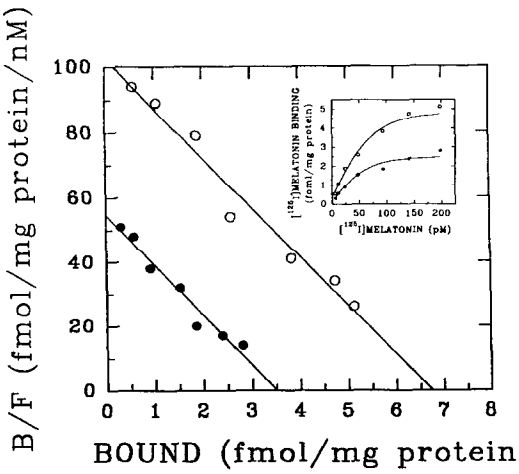


Fig. 8. Scatchard plots showing the stress-induced decrease in melatonin receptor density in the SCN. Means of triplicate values from one of three separate experiments conducted in triplicate are presented. Control (○), stress (●).

over, saturation binding studies (Fig. 5) indicated a decline in the density of high-affinity sites at 19 30h (30 min before the onset of darkness), when light-induced suppression of melatonin production was still evident. These findings suggest that other factors, in addition to melatonin levels, also influence the rhythmicity in high-affinity binding. The decrease in the concentration of high-affinity sites coincided with the appearance of a low-affinity site, whose density increased throughout the dark phase, while that of the high-affinity site continued to decrease. It is now known that the high-affinity receptor for melatonin is converted to a low-affinity state in the presence of guanine nucleotides and/or monovalent cations (Niles, 1990; Ying and Niles, 1991). Moreover, these receptors mediate the inhibition of adenylate cyclase activity by melatonin (Niles and Hashemi, 1990b; Ying et al., 1992), via a pertussis toxin-sensitive G_i protein (Carlson et al., 1989; Niles et al., 1991). Therefore, it appears that the two sites mentioned above, represent the high-affinity and low-affinity states of the melatonin receptor. This view is supported by the conversion of all high-affinity sites to a low-affinity state in the presence of GTP and sodium as indicated in Fig. 6.

The foregoing raises the question of why low-affinity binding appears only at the times indicated, while high-affinity binding is present throughout the circadian cycle. An interesting possibility is that there is a circadian rhythm in the density of the regulating G_i protein. There is evidence that pertussis toxin-sensitive G_i and other G proteins can be down-regulated following exposure of their associated receptors to agonists (Green, 1987; Milligan and Green, 1991). Therefore, it is conceivable that G_i levels in the SCN may be elevated during the day, when agonist (melatonin) levels are low, and progressively down-regulated with increasing synthesis and secretion of melatonin at night. Since G_i is essential for converting melatonin receptors to a high-affinity state (Carlson et al., 1989; Niles et al., 1991), high levels of this G protein during the day may account for the absence of low-affinity binding during most of the light phase. Conversely, an agonist-induced decrease in G_i levels during darkness would allow detection of low-affinity sites. However, in addition to agonist concentrations, neurotransmitters and other agents in the SCN may influence G_i levels and receptor affinity, since, as noted above, the decline in high-affinity binding, with the first appearance of low-affinity sites, occurred late in the light phase when circulating melatonin levels were still low. Additional studies are required to determine the mechanisms involved in regulating the affinity of the melatonin receptor in the SCN, where this hormone is thought to play a role in modulating circadian rhythmicity.

In the only other study of high-affinity diurnal binding of [^{125}I]MEL in the SCN, Laitinen et al (1989) reported several observations which are similar to our findings, with one notable exception. These investigators, like us, found no differences in saturation binding in coronal slices taken throughout the rostro-caudal length of the SCN. Also in agreement with the present study, they detected a single high-affinity site early in the light phase, while both high- and low-affinity sites were detected at the light-dark transition. However, in marked contrast to our finding that high-affinity binding is highest late in the light period and lowest during darkness, they reported almost exactly the inverse with their highest binding occurring around the dark-light transition and their trough at the end of the light phase (Laitinen et al., 1989). The reason for this major discrepancy is not clear at present. While we measured binding in homogenates prepared from sections of Sprague-Dawley rat hypothalami, the above-mentioned group used quantitative autoradiography in coronal sections from Wistar rat brain. Binding parameters agree reasonably well between the two studies, so it is unlikely that these experimental differences produced the above mentioned discrepancy. Although unlikely, the possibility that strain-related differences in diurnal binding are involved, cannot be ruled out at this time.

Agonist-induced down-regulation of receptors is a well-documented mechanism which allows target cells to adapt to high levels of hormones or other endogenous agents (Sibley and Lefkowitz, 1985). Thus, an increase in circulating melatonin during darkness or after swim-stress caused a significant reduction in the density of high-affinity receptors in the SCN. Conversely, prolonged depletion of circulating melatonin resulted in a significant increase in receptor density late in the light period, suggesting that binding sites are maximally sensitive at that time. The increased sensitivity of melatonin receptors after several hours of

exposure to light is consistent with several reports that the physiological effects of this hormone are time dependent. For example, melatonin was found to suppress reproductive activity in hamsters only when injected later than 6.5 h after the start of the photoperiod (Chen et al., 1980). However, the subcutaneous implantation of a high dose of melatonin blocked its own antigonadotrophic effects in blinded and anosmic rats by presumably maintaining receptors in a down-regulated and desensitized state (Chen and Reiter, 1980). In more recent studies, melatonin was significantly more potent in depressing LHRH-stimulated cyclic nucleotide accumulation in rat pituitaries removed at the light-dark transition than at earlier times in the light cycle (Vanecek and Vollrath, 1990). Similarly, it was most effective in suppressing the firing rate of SCN neurons when iontophoretically applied at times around the day-night transition (Stehle et al., 1989). Furthermore, melatonin can reset SCN electrical activity in vitro, when applied to hypothalamic slices, late in the circadian cycle to which donor rats had been exposed (McArthur et al., 1991).

In a diurnal study of [^{125}I]MEL binding in rat hypothalamic membranes, Zisapel et al. (1988), reported the presence of a low-affinity site which exhibited maximal density during the light phase with a sharp decrease in binding during darkness, but no changes in affinity. The binding affinity ($K_d = \sim 80$nM) of that site is about 1500-fold lower than that of the picomolar-affinity site described in this report, making any meaningful comparisons difficult.

The rapid change in melatonin receptor density, with no changes in binding affinity, following swim-stress, is similar to that reported for CNS benzodiazepine receptors in animals exposed to acute stress (Medina et al., 1983; Andrews et al., 1992). It is unlikely that the decrease in [^{125}I]MEL binding is due to residual melatonin present in the brains of stressed animals, as tissues were washed extensively before use in assays. Instead, the apparent stress-induced down-regulation of binding sites may be due to rapid modification and/or internalization of receptors in the SCN following occupation by melatonin. There is evidence that receptors can undergo rapid agonist-induced internalization within minutes (Knutson et al., 1983) thus, the time of 30 minutes, from the start of stress to sacrifice, was sufficient for receptor down-regulation to have occurred. However, it should be noted that other stress-induced changes might have been involved in altering [^{125}I]MEL binding.

In summary, the diurnal rhythmicity of melatonin receptor density suggests that high affinity binding sites in the SCN should be maximally responsive to this hormone late in the light phase, up to 1–2 h before the light-dark transition point. The inverse relationship between hormone levels and binding, under two different physiological conditions, indicates that melatonin plays a role in regulating its own target sites in the SCN.

Acknowledgements

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