Brain Research, 565 (1991) 158–161

ADONIS 0006899391249353

BRES 24935

Short Communications

Melatonin directly resets the rat suprachiasmatic circadian clock in vitro

Angela J. McArthur[1], Martha U. Gillette[1,2,3] and Rebecca A. Prosser[3,*]

[1]*Department of Physiology and Biophysics,* [2]*Department of Cell and Structural Biology and* [3]*The Neuroscience Program, University of Illinois, Urbana, IL 61801 (U.S.A.)*

(Accepted 20 August 1991)

Key words: Brain slice; Melatonin; Phase-shift; Circadian rhythm; Suprachiasmatic nucleus; Pacemaker

The environmental photoperiod regulates the synthesis of melatonin by the pineal gland, which in turn induces daily and seasonal adjustments in behavioral and physiological state. The mechanisms by which melatonin mediates these effects are not known, but accumulating data suggest that melatonin modulates a circadian biological clock, either directly or indirectly via neural inputs. The hypothesis that melatonin acts directly at the level of the suprachiasmatic nucleus (SCN), a central mammalian circadian pacemaker, was tested in a rat brain slice preparation maintained in vitro for 2–3 days. Exposure of the SCN to melatonin for 1 h late in the subjective day or early subjective night induced a significant advance in the SCN electrical activity rhythm; at other times melatonin was without apparent effect. These results demonstrate that melatonin can directly reset this circadian clock during the period surrounding the day–night transition.

Melatonin is produced by the pineal gland during the dark phase of the diurnal cycle[19,25]. In mammals this daily (circadian) rhythm of synthesis is regulated by a multisynaptic pathway from an endogenous biological clock in the suprachiasmatic nuclei (SCN) of the hypothalamus[9,16]. Light, which modulates the SCN via the retina and retinohypothalamic tract[12,13], entrains the melatonin rhythm by suppressing its synthesis during the day.

Recent work suggests that melatonin may feed back upon the circadian oscillator that times its rhythmic appearance. Acute administration of melatonin can entrain activity rhythms of SCN-intact rodents in constant photic conditions[1,18]. The entraining effects of melatonin are limited to a discrete time of the day corresponding to the hours surrounding the day-night transition.

Anatomical and physiological evidence strengthens the possibility that these integrative effects may be exerted directly on the circadian clock. The SCN is one of only two central nervous system loci with high levels of melatonin binding sites[20,26–28]. The density of melatonin binding sites in the SCN exhibits circadian variation[11,29]. Also, melatonin injections in rodents induce acute changes in SCN glucose utilization and protein synthesis when administered in late day[2,21]. Finally, SCN neuronal activity in brain slices is acutely depressed by melatonin application only during late subjective day[14,22,24]. The relationship between these observations and SCN circadian clock function has not been tested.

We addressed the question of whether melatonin can directly modulate SCN time-keeping by examining its ability to change the phase of an endogenous circadian rhythm. Coronal hypothalamic brain slices (500 μm thick) were prepared during the day from 7- to 8-week-old male Long–Evans rats raised in our inbred colony and housed from birth on a 12:12 h light–dark (LD) schedule. The brain slices were maintained at 37 °C in a Hatton-style brain slice dish under constant illumination[3]. The tissue was continuously perfused with glucose/bicarbonate-supplemented Earle's balanced salt solution (EBSS, Gibco) at pH 7.2 and saturated with 95% O_2:5% CO_2.

Melatonin (Sigma) was dissolved at 10^{-3} M in 95% EtOH within 0.5 h of use and serially diluted to 10^{-9} M with fresh medium. This concentration is within one order of magnitude above the lowest saturation point of 2-^{125}I-melatonin binding to rat SCN[11]. Perfusion was stopped and the medium in the brain slice chamber was completely replaced with the melatonin/EBSS solution for 1 h, after which time the melatonin solution was exchanged with normal medium and perfusion was resumed.

We have established that SCN in our brain slice system sustain a spontaneous, regular circadian rhythm of single unit neuronal activity for up to 3 days in vitro[4,17]. This coincides with the rhythm of multiunit neuronal activity recorded in vivo[7,8]. The pattern of SCN activity is

* Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305, U.S.A.
Correspondence: M.U. Gillette, Department of Cell and Structural Biology, University of Illinois, 505 S. Goodwin Ave., Urbana, IL 61801, U.S.A.

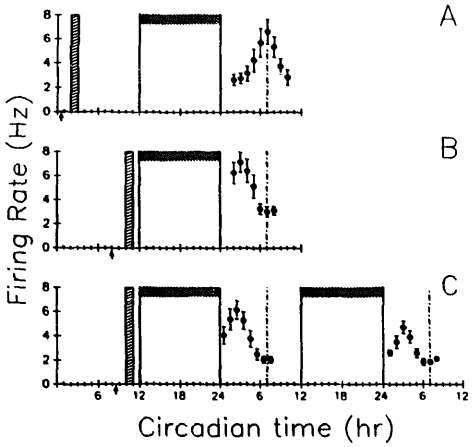

Fig. 1. Effects of melatonin on the time-of-peak in the SCN electrical activity rhythm depend upon the circadian time of treatment. Results of 3 individual experiments are plotted as the running 2-h mean firing rates vs. circadian time. A: melatonin applied at CT 2.0 had no detectable effect on the time of peak activity. B: melatonin treatment at CT 10.0 advanced the phase of the neuronal activity rhythm nearly 4 h, such that the peak on day 2 in vitro occurred at CT 3.0. C: recording on days 2 and 3 in vitro after melatonin treatment at CT 10.0 on day 1 revealed that the peak occurred at CT 3.0 on both days indicating a permanent resetting of the clock. Dashed line indicates mean time-of-peak in untreated slices (CT 6.9 ± 0.2, n = 8), as well as EtOH controls (CT 7.0, n = 2). Horizontal bar indicates subjective night. Treatment period with 10^{-9} M melatonin is indicated with a vertical bar. Arrow indicates time of slice preparation.

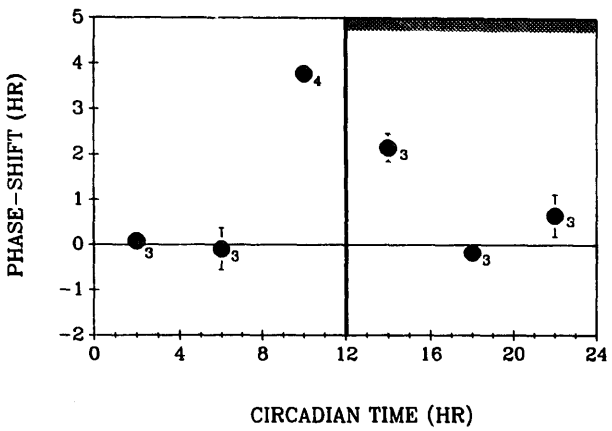

Fig. 2. Phase-response curve for melatonin. The effect of melatonin application at various circadian times (CT 2,6,10,14,18,22) was tested in at least 3 separate experiments per time point (subscript indicates number of experiments). The average magnitudes of phase-shifts ± S.E.M. are plotted according to the circadian time at which the 1-h melatonin pulse was initiated. Treatment near the entrained day-night transition (vertical line indicates time of lights-off, horizontal bar indicates subjective night) resulted in a significant phase-advance in the peak in the circadian rhythm of electrical activity, whereas treatment at other times had no apparent effect.

characteristically high during the day and low at night[3,6]. The electrical activity of the ensemble of melatonin-treated SCN neurons was monitored extracellularly during day 2 and/or 3 in vitro in order to assess the time of peak activity, a reliable measure of phase of the SCN clock[4,5]. This study examined SCN sensitivity to melatonin at six different time points over the circadian cycle.

The phase of the circadian cycle at which the treatment was applied determined the effect of melatonin on the circadian rhythm of neuronal activity. Melatonin treatment of SCN at circadian time 2.0 (CT 2 = 2.0 h after 'lights on' in the 24 h lighting schedule in the rat colony) on the first day in vitro had no effect on the time-of-peak in the electrical activity rhythm on the second day. The time of peak activity in these experiments was CT 7.0 ± 0.1, n = 3, (Fig. 1A), which is not significantly different from the time-of-peak in untreated slices on day 2 (CT 6.9 ± 0.2, n = 8)[17].

In contrast, when the melatonin pulse was given at CT 10.0, it significantly advanced the phase of the neuronal activity rhythm (Fig. 1B). On the day after melatonin treatment, the oscillation peaked near CT 3.0, almost four hours earlier than untreated controls (phase advance (ϕ_A) = 3.8 ± 0.1 h, n = 4), or controls treated at CT 10.0 for 1 h with 0.1% EtOH/EBSS (CT 7.0, n = 2). When the time-of-peak was examined on day 3 following melatonin treatment at CT 10.0 on day 1, the phase-advance was maintained (ϕ_A = 3.8 ± 0.4 h, n = 3, Fig. 1C). Although the amplitude of the peak was dampened (not uncommonly observed in 3 day experiments)[17], the magnitude of the shift was stable over this period. This indicates that the underlying clock mechanism is permanently reset during the first cycle after melatonin treatment and thereafter oscillates normally from its new phase.

The suprachiasmatic clock appears to be sensitive to melatonin treatment only during the period surrounding the day-night transition of the entrained lighting cycle. The results of 19 experiments examining the effect of exogenous melatonin at six times across the circadian cycle are plotted in Fig. 2. Melatonin had no significant effect on the phase of the electrical activity rhythm except at late day (CT 10.0, ϕ_A = 3.8 h ± 0.1, n = 4, $P <$ 0.0001, Student's t-test) and early night (CT 14, ϕ_A = 2.0 h ± 0.2, n = 3, $P <$ 0.0001).

This temporal pattern of sensitivity of the rat SCN to melatonin differs from the timing of sensitivity to serotonin: the SCN rhythm is reset by melatonin only at the period surrounding dusk, while serotonin causes phase resetting during mid-day (peak sensitivity at CT 7) and is ineffective at CT 13.0[15]. Serotonin (5-hydroxytryptamine) is a precursor of melatonin (5-methoxy-*N*-acetyltryptamine) and binds to melatonin receptors with low affinity[23]. However, the two compounds differ at the moiety responsible for stimulating the functional response at melatonin binding sites[23]. The fact that these

two indoleamines affect the SCN clock during distinctly different phases implies that the effect of melatonin near the day–night transition is specific, based upon key structural determinants of the melatonin molecule.

Specificity of the melatonin effect is further supported by experiments with the potent melatonin agonist, 2-iodo-melatonin (2-I-melatonin) (Research Biochemicals Inc.). A 1 h exposure to 10^{-9} M 2-I-melatonin early in the day, at CT 2.0, had little effect on the subsequent cycle of electrical activity ($\phi_{Delay} = 0.6$ h, $n = 1$), whereas treatment at CT 10.0, a time at which melatonin is highly effective, phase advanced the time-of-peak nearly 7 h ($\phi_A = 6.8$ h $\pm$ 0.1, $n = 3$). The greater efficacy of 2-I-melatonin at the melatonin-sensitive time is consistent with its higher specific activity at melatonin binding sites. These results complement the finding that the SCN is insensitive to serotonin treatment at dusk and strengthen the case that the effects of melatonin on the SCN are specific.

The concordance in temporal sensitivity to melatonin between in vivo data on entrainment of behavioral rhythms and our in vitro data on phase-shifting of SCN electrical activity rhythm implies that melatonin acts directly upon the SCN in the organism. We found significant phase advances in the electrical activity rhythm of the SCN when treated with melatonin or 2-I-melatonin during the late day or early evening. This period coincides with the time of maximum density in low-affinity melatonin binding sites in rat SCN[11], supporting our finding of a temporal window of sensitivity to melatonin in the SCN around dusk in this species. Nocturnal melatonin synthesis in the rat does not increase until about 2 h after the onset of darkness[10]; however, our results suggest that melatonin can function as an input to the clock at this time. Thus, melatonin synthesis is regulated by SCN output and SCN output is, in turn, modulated by melatonin in a classic feedback loop. The organization of this regulatory loop suggests that melatonin synchronizes the animal to the time of nightfall by daily adjusting the SCN circadian clock. This feedback may contribute to seasonal adjustments in circadian rhythms with changing night length over the course of the year.

The authors wish to thank Drs. E. Gallman, R. Gillette, G. Jackson, and E. Satinoff for helpful comments on the manuscript. Support of NINDS Grant NS-22155 to M.U.G. and NIH Grant 5T32GM07143 to A.J.M. is gratefully acknowledged. Preliminary results were presented to the Society for Neuroscience (A.J. McArthur, R.A., Prosser, and M.U. Gillette, *Soc. Neurosci. Abstr.*, 15 (1989) 1059).

1 Cassone, V.M., Chesworth, M.J. and Armstrong, S.M., Entrainment of rat circadian rhythms by daily injection of melatonin-depends upon the hypothalamic suprachiasmatic nuclei, *Physiol. Behav.*, 36 (1986) 1111–1121.
2 Cassone, V.M., Roberts, M.H. and Moore, R.Y., Effects of melatonin on 2-deoxy-[1-^{14}C]glucose uptake within rat suprachiasmatic nucleus, *Am. J. Physiol.*, 255 (1988) R332–R337.
3 Gillette, M.U., The suprachiasmatic nuclei: circadian shifts induced at the time of hypothalamic slice preparation are preserved in vitro, *Brain Research*, 379 (1986) 176–181.
4 Gillette, M.U. and Prosser, R.A., Circadian rhythm of the rat suprachiasmatic brain slice is rapidly reset by daytime application of cAMP analogs, *Brain Research*, 474 (1988) 348–352.
5 Gillette, M.U. and Reppert, S.M., The hypothalamic suprachiasmatic nuclei: circadian patterns of vasopressin secretion and neuronal activity in vitro, *Brain Res. Bull.*, 19 (1987) 135–139.
6 Green, D.J. and Gillette, R., Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic slice, *Brain Research*, 245 (1982) 198–200.
7 Inouye, S.-I.T. and Kawamura, H., Persistence of circadian rhythmicity in a mammalian hypothalamic 'island' containing the suprachiasmatic nucleus, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5962–5966.
8 Inouye, S.-I.T. and Kawamura, H., Characteristics of a circadian pacemaker in the suprachiasmatic nucleus, *J. Comp. Physiol.*, 146 (1982) 153–160.
9 Klein, D.C., In D. Evered and S. Clark (Eds.), *Photoperiodism, Melatonin, and the Pineal, Ciba Foundation Symposium 117*, Pittman, London, 1985, p. 38.
10 Klein, D.C. and Weller, J.L., Indole metabolism in the pineal gland: a circadian rhythm of *N*-acetyltransferase, *Science*, 169 (1970) 1093–1095.
11 Laitinen, J.T., Castren, E., Vakkuri, O. and Saavedra, J.M., Diurnal rhythm of melatonin binding in the rat suprachiasmatic nucleus, *Endocrinology*, 124 (1989) 1585–1587.
12 Lewy, A.J., Wehr, T.A., Goodwin, F.K. and Newsome, D.A., Light suppresses melatonin secretion in humans, *Science*, 210 (1980) 1267–1269.
13 Lincoln, G.A., Ebling, F.J.P. and Almeida, O.F.X. In D. Evered and S. Clark (Eds.), *Photoperiodism, Melatonin, and the Pineal, CIBA Foundation Symposium 117*, Pittman, London, 1985, p. 129.
14 Mason, R. and Brooks, A., The electrophysiological effects of melatonin and a putative melatonin antagonist (*N*-acetyltryptamine) on rat suprachiasmatic neurones in vitro, *Neurosci. Lett.*, 95 (1988) 296–301.
15 Medanic, M. and Gillette, M.U., Serotonin phase shifts the circadian rhythm of electrical activity in the rat SCN in vitro, *Soc. Neurosci. Abstr.*, 16 (1990) 771.
16 Moore, R.Y. and Klein, D.C., Visual pathways and the central neural control of a circadian rhythm in pineal serotonin *N*-acetyltransferase activity, *Brain Research*, 71 (1974) 17–33.
17 Prosser, R.A. and Gillette, M.U., The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP, *J. Neurosci.*, 9 (1989) 1073–1081.
18 Redman, J., Amstrong, S.M. and Ng, K.T., Free-running activity rhythms in the rat: entrainment by melatonin, *Science*, 219 (1983) 1089–1091.
19 Reiter, R.J. (Ed.), *The Pineal Gland*, Raven, New York, 1984.
20 Reppert, S.M., Weaver, D.R., Rivkees, S.A. and Stopa, E.G., Putative melatonin receptors in a human biological clock, *Science*, 242 (1988) 78–81.
21 Roberts, M.H., Cassone, V.M. and Moore, R.Y., Effects of melatonin on suprachiasmatic nucleus metabolism in rats, *Soc. Neurosci. Abstr.*, 13 (1987) 1038.
22 Shibata, S., Cassone, V.M. and Moore, R.Y., Effects of melatonin on neuronal activity in the rat suprachiasmatic nucleus in vitro, *Neurosci. Lett.*, 97 (1989) 140–144.
23 Stankov, B. and Reiter, R., Melatonin receptors: current status, facts, and hypotheses, *Life Sci.*, 46 (1990) 971–982.

24 Stehle, J., Vanecek, J. and Vollrath, L., Effects of melatonin on spontaneous electrical activity of neurons in rat suprachiasmatic nuclei: an in vitro iontophoretic study, *J. Neural Transm.*, 78 (1989) 173–177.
25 Underwood, H. and Goldman, B.D., Vertebrate circadian and photoperiodic systems: role of the pineal gland and melatonin, *J. Biol. Rhythms*, 2 (1987) 279–316.
26 Vanecek, J., Melatonin binding sites, *J. Neurochem.*, 51 (1987) 1436–1440.
27 Vanecek, J., Pavlik, A. and Illnerova, H., Hypothalamic melatonin receptor sites revealed by autoradiography, *Brain Research*, 435 (1987) 359–362.
28 Weaver, D.R., Rivkees, S.A. and Reppert, S.M., Localization and characterization of melatonin receptors in rodent brain by in vitro autoradiography, *J. Neurosci.*, 9 (1989) 2581–2590.
29 Zisapel, N., Nir, I. and Laudon, M., Circadian variation in melatonin binding sites in discrete areas of the male rat brain, *FEBS Lett.*, 232 (1988) 172–176.