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Melatonin and circadian control in mammals

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Summary. Although pinealectomy has little influence on the circadian locomotor rhythms of laboratory rats, administration of the pineal hormone melatonin has profound effects. Evidence for this comes from studies in which pharmacological doses of melatonin are administered under conditions of external desynchronization, internal desynchronization, steady state light-dark conditions, and phase shifts of the zeitgeber. Taken together with recent findings on melatonin receptor concentration in the rat hypothalamus, particularly at the level of the suprachiasmatic nuclei, these results suggest that melatonin is a potent synchronizer of rat circadian rhythms and has a direct action on the circadian pacemaker. It is possible, therefore, that the natural role of endogenous melatonin is to act as an internal zeitgeber for the total circadian structure of mammals at the level of cell, tissue, organ, whole organism and interaction of that organism with environmental photoperiod changes.
Key words. Melatonin; synchronization; phase adjustment; photoperiod; receptors; phylogeny; ontogeny; circadian rhythms; zeitgeber.

Introduction

As with other vertebrates, investigations into the function of the mammalian pineal body have concentrated primarily on the role played by the chemical melatonin. Melatonin is released into the general circulation during the hours of darkness, irrespective of whether the species is nocturnal or diurnal in its behavioural activity pattern.

Measurement of pineal N-acetyltransferase activity, the enzyme which drives the pineal melatonin rhythm, shows that it directly approximates circadian output from the suprachiasmatic nuclei (SCN) located in the anterior hypothalamus. The SCN are the major central nervous system (CNS) pacemakers responsible for generating circadian rhythmicity in mammals and are entrained to the external light-dark (LD) cycle by their own retinohypothalamic pathway. Therefore, while the pineal and SCN are anatomically distant and are connected by a circuitous route, the argument can be made that melatonin release is functionally part of SCN output[6]. Because of its periodic release and its mirroring of SCN metabolic activity, melatonin is the ideal circadian chemical messenger to act as an internal zeitgeber, thereby imposing synchronicity on the multitude of daily rhythms at the level of cell, tissue and organ, and thereby preventing internal desynchrony[5,6].

Nature has selected melatonin to fulfil different synchronization roles and thereby solve different synchronization problems in different species. However, while synchronization problems in temporal organization may seem superficially different, essentially the same strategy can be used to solve them. From cell cycles to seasonal cycles a daily periodic signal is needed within the internal milieu to carefully to adjust phase between critical rhythms at all levels of temporal organization. The most researched example of this is found in photoperiodic time measurement of seasonally breeding, photosensitive mammalian species, where melatonin's action as an internal zeitgeber can be described in terms of internal or external coincidence models depending upon the species[27] (Bartness and Goldman; Ebling and Foster, this issue).

Below, after a consideration of the results of pinealectomy (Px) studies, the evidence for internal zeitgeber properties of melatonin in mammals is marshalled. The bulk of this evidence comes from studies on laboratory rats and this is reviewed together with crucial findings of melatonin receptor location in the hypothalamus. Finally, alternative explanations are considered.

The pineal and synchronicity; effects of pinealectomy

Pinealectomy has little effect on rat or hamster free-running locomotor rhythms in constant light (LL) or constant dark (DD) (see Armstrong[6] for references). It is reasonable to conclude that the pineal body is not involved in the generation of rodent circadian locomotor rhythms although this cannot be generalized to all mammals until a greater variety of species has been studied. Nevertheless, the commonly drawn conclusion that the pineal body should be relegated to a minor role in the mammalian circadian system does not necessarily follow. Removal of an organ is not always the most instructive way of assessing its biological importance:

1) Laboratory rats survive remarkably well without an adrenal cortex and, indeed, without adrenal glands, providing that rats are maintained on a saline drinking solution; 2) for years the thymus gland was deemed to be vestigial simply because it had no obvious function; 3) the circadian locomotor rhythm of chickens is unaffected by Px and yet the bulk of bird pineal-circadian rhythm biochemistry is carried out on chickens. (Interestingly, these results are then generalized to other species such as the sparrow, where circadian rhythmicity in constant conditions is affected by Px.)

The crucial difference between the chicken pineal and that of the adult rat is that the former shows endogenous rhythmicity when studied in vitro, and can therefore be conceptualized as a biological clock in its own right. Although the lack of in vivo rhythmicity in the rat may represent a problem of technology, this is unlikely as the rat pup shows endogenous pineal rhythmicity which ceases about the time that neural connections to the SCN are completed (see Wainwright[39] for references).

In the present scheme it is envisaged that 1) endogenous rhythmicity is not necessarily a property of the adult mammalian pineal gland, but that 2) the SCN drive the pineal melatonin rhythm, so that 3) circulating melatonin is the main circadian periodic signal to the rest of the organism, thereby 4) synchronizing other daily bodily rhythms that may be exogenous, slave or even self-sustained[5,6]. The effectiveness of melatonin as a synchronizing agent is dependent upon 5) the concentration of receptors in the target tissue, and these may vary with such factors as time of day, season, gonadal status, developmental stage and age. It also follows that rat Px should produce internal desynchrony and the evidence for this has been assessed elsewhere[6], although the crucial experiments have yet to be conducted.

Melatonin, gating and synchronicity

Daily injections of melatonin over a range of doses[11] entrain rat free-running, circadian locomotor rhythms in DD[26]. Entrainment takes place when the onset of activity has just passed through injection time so that the active phase (α) follows injection while the quiescent phase (ϱ) precedes it (fig. 1 A and B). In these rats tau (τ) is longer than 24 h and melatonin induces entrainment by exerting small phase advances[8] at approximately CT 10.5 (fig. 1 C). There is thus a 'narrow window of sensitivity' or 'gate of circadian frequency' at which the pacemaker is responsive to exogenous melatonin and phase-trapping occurs. Melatonin may be classified as an impulse zeitgeber[6,10]. If τ is too long, entrainment may not occur[14,36] at least by this method or route of melatonin administration. The phase of entrainment in the nocturnally active rat is 180° removed from that of the diurnally active starling[18] and a diurnal species of lizard (see Underwood, this issue), thus mimicking the natural-

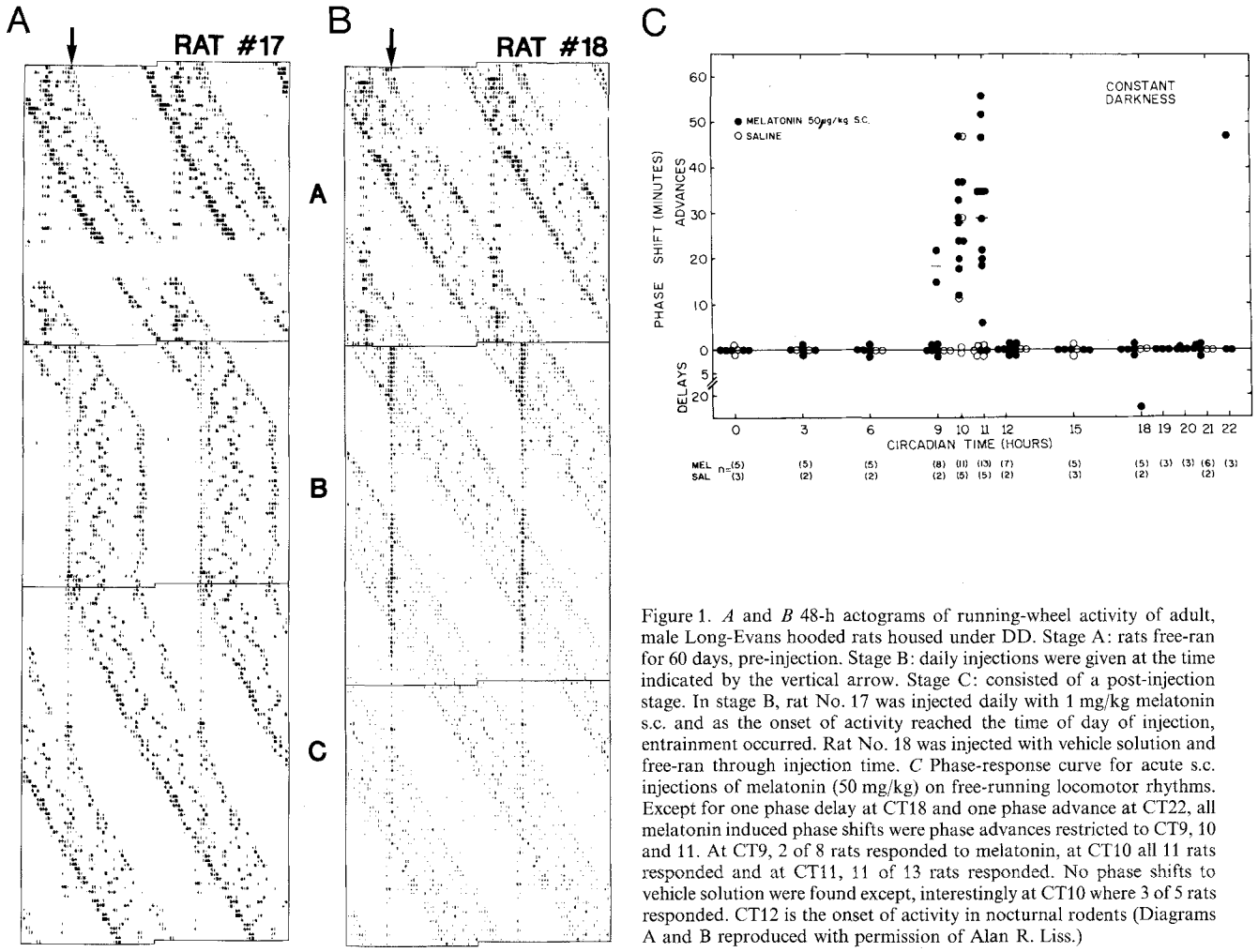

Figure 1. *A* and *B* 48-h actograms of running-wheel activity of adult, male Long-Evans hooded rats housed under DD. Stage A: rats free-ran for 60 days, pre-injection. Stage B: daily injections were given at the time indicated by the vertical arrow. Stage C: consisted of a post-injection stage. In stage B, rat No. 17 was injected daily with 1 mg/kg melatonin s.c. and as the onset of activity reached the time of day of injection, entrainment occurred. Rat No. 18 was injected with vehicle solution and free-ran through injection time. *C* Phase-response curve for acute s.c. injections of melatonin (50 mg/kg) on free-running locomotor rhythms. Except for one phase delay at CT18 and one phase advance at CT22, all melatonin induced phase shifts were phase advances restricted to CT9, 10 and 11. At CT9, 2 of 8 rats responded to melatonin, at CT10 all 11 rats responded and at CT11, 11 of 13 rats responded. No phase shifts to vehicle solution were found except, interestingly at CT10 where 3 of 5 rats responded. CT12 is the onset of activity in nocturnal rodents (Diagrams A and B reproduced with permission of Alan R. Liss.)

ly occurring covariation between endogenous melatonin release and behavioural activity.

The internal synchronizing properties of melatonin are best exemplified by findings of the reorganization of severely disrupted activity rhythms by daily melatonin injections (splitting, multiple ultradian components or apparent total arrhythmicity) after subjection of male rats to an exotic lighting regime and bright LL[14]. The favoured interpretation is that melatonin acts upon the coupling or phase relationship between several endogenous oscillators which make up the CNS circadian pacemaker.

Under steady state LD conditions, late afternoon injections of melatonin phase advance rat locomotor activity rhythms which phase lag dark onset, providing that the phase angle difference is not more than 3 h[7,9] (fig. 2C and D). The pineal has long been thought to be involved in the re-entrainment process[6,24]. After phase shifts of the zeitgeber by 5- or 8-h phase advances, daily melatonin injections can alter the direction, rate of, or have no effect upon re-entrainment depending upon clock time of injection in relation to the original or newly shifted LD cycle[25] (fig. 2A and B).

In short, exogenous melatonin has profound effects on the circadian system of rats under conditions of external desynchronization, internal desynchronization, steady state LD cycles and phase shifts of the zeitgeber.

Melatonin target sites

The biochemical action of melatonin on the hypothalamus is reviewed elsewhere (see Williams and Morgan, this issue) but certain aspects need to be considered here. The density of low affinity binding of ^{125}I-iodomelatonin to rat brain synaptosomes shows a distinct daily rhythm in the hypothalamus, peaking late in the light period, but no rhythm is apparent in the striatum. High affinity binding within the SCN has also been reported to exhibit a variation over 24 h. This regional difference indicates that the rhythmicity in concentration of melatonin receptors is not due to down-regulation by elevated nocturnal circulating melatonin[42]. The fact that total hypothalamic melatonin receptors peak late in the photophase may explain why entrainment by exogenous melatonin and phase advances are limited to this time: receptor concentration represents a functional gating mechanism. How-

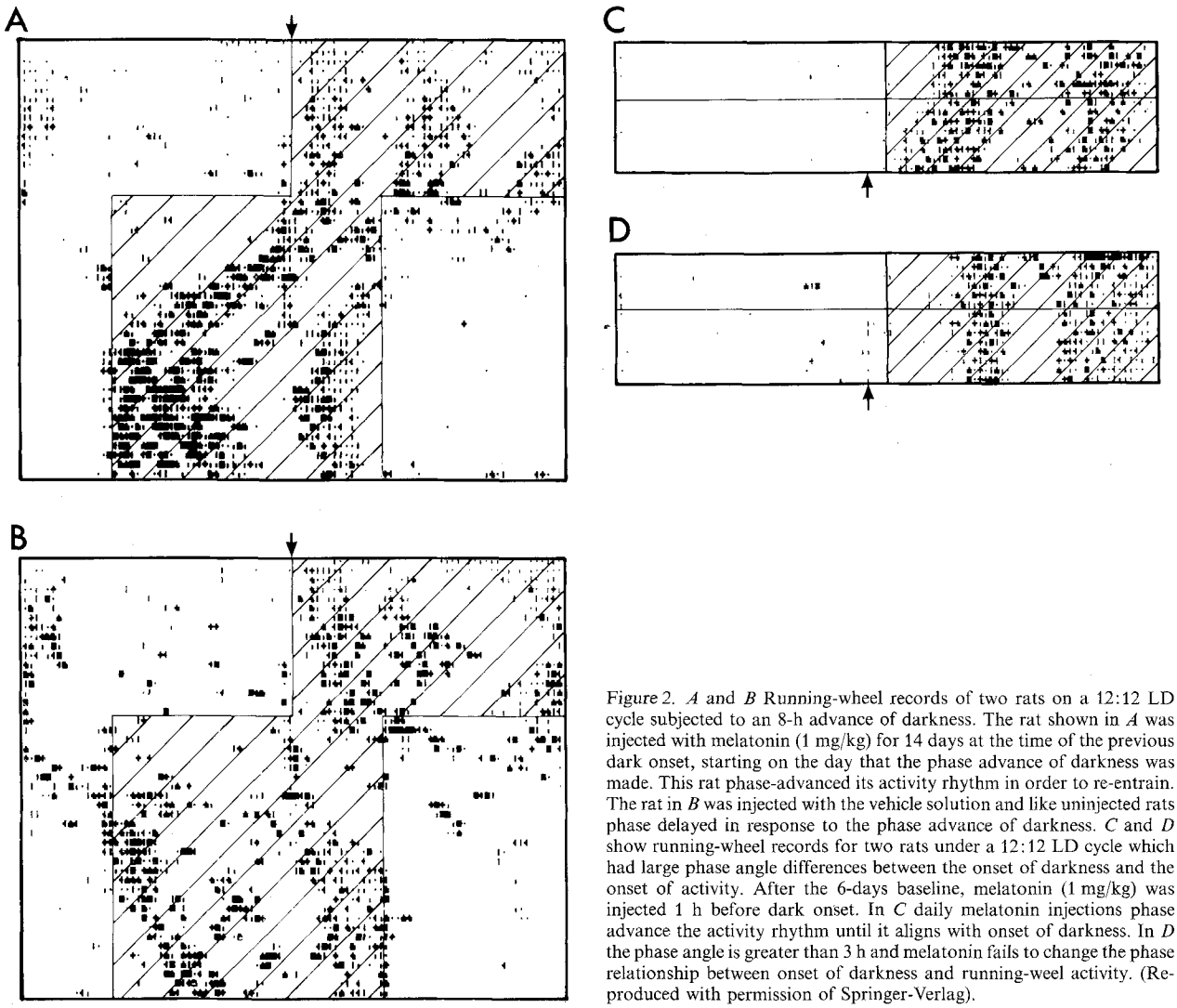

Figure 2. *A* and *B* Running-wheel records of two rats on a 12:12 LD cycle subjected to an 8-h advance of darkness. The rat shown in *A* was injected with melatonin (1 mg/kg) for 14 days at the time of the previous dark onset, starting on the day that the phase advance of darkness was made. This rat phase-advanced its activity rhythm in order to re-entrain. The rat in *B* was injected with the vehicle solution and like uninjected rats phase delayed in response to the phase advance of darkness. *C* and *D* show running-wheel records for two rats under a 12:12 LD cycle which had large phase angle differences between the onset of darkness and the onset of activity. After the 6-days baseline, melatonin (1 mg/kg) was injected 1 h before dark onset. In *C* daily melatonin injections phase advance the activity rhythm until it aligns with onset of darkness. In *D* the phase angle is greater than 3 h and melatonin fails to change the phase relationship between onset of darkness and running-weel activity. (Reproduced with permission of Springer-Verlag).

ever, the relationship is not a clear one, because recent reports using autoradiographic determinations of melatonin binding within the SCN suggest that receptor levels in this area are in fact lower during the late photophase (Morgan and Williams, this issue).

By the use of ^{125}I-iodomelatonin autoradiography, consistent and intense binding is found in the SCN of rats, Djungarian hamsters and humans[30, 38, 40]. Although earlier studies have been inconsistent (D. R. Weaver, personal communication), more recently it has been clearly established that the SCN of the adult Syrian hamster binds ^{125}I-iodomelatonin (Morgan and Williams, this issue). However, adult Syrian hamsters fail to entrain to melatonin injections (Ellis and Turek cited in Turek et al.[37]; Menaker et al. cited in Armstrong and Redman[9]). Two groups of researchers have suggested independently that the melatonin entrainment effect may be governed by the previous photoperiodic and thus gonadal history[34] (W. Puchalski, personal communication). Given that hypothalamic melatonin receptor concentrations have been reported to vary with ovariectomy and castration[43], it is possible that responsiveness to melatonin in the Syrian hamster may be induced by appropriate photic, and hence gonadal, manipulations.

The intense concentration of high affinity melatonin receptors in the SCN make it likely that exogenous melatonin produces entrainment via this structure. Previously, it has been shown that removal of the pineal gland or retina (Chesworth, unpublished data) or chemical lesions to the brain catecholamine and monoamine systems[12] do not interfere with entrainment by melatonin, while SCN lesions do abolish entrainment[12]. In addition, melatonin interferes with the daily rhythm in metabolic activity of the SCN as indexed by labelled 2-deoxy-glucose autoradiography[13], and the most sensitive time is late in the subjective day, corresponding to the phase of sensitivity to melatonin entrainment effects.

Taking all of the above together, it is likely that exogenous melatonin affects the rat circadian system by direct stimulation of the SCN. The SCN appears to have a gate

or narrow window of sensitivity by which melatonin can trap circadian locomotor rhythms at a certain circadian phase. The functional importance of this can only be speculated upon at present.

Photoperiod, gating and phase adjustment

The presence of a high density of melatonin receptors in the SCN indicates the feedback of melatonin onto the CNS pacemaker. Since the two oscillator models of non-parametric entrainment[21] seem to account entirely for rodent circadian and seasonal photoperiodic changes, the existence of melatonin feedback is perplexing. One possible explanation lies in the observation that many species of rodent and marsupial, when free-running in constant laboratory condition, have a very precise onset to α while the end is ragged and terminates several hours before lights on. If this pattern is present in nature there is a distinct possibility that many individuals of a burrowing species would rarely see dawn, particularly under long scotoperiods. In the situation where the dawn light signal (and thus the phase advance stimulus) is absent and τ is longer than 24 h, the pacemaker would tend to phase delay. Melatonin release coinciding with a pacemaker gate at around CT 10–12 would counter this delay by inducing a phase advance. This mechanism would enable the organisms to track dark onset accurately. Single dark pulses phase advance golden hamster circadian rhythms in LL at around CT 10 to CT 12[16]. Although there is preliminary data that 3-h dark pulses are effective in rats (unpublished data) it is not yet established whether melatonin is released during such pulses.

A similar suggestion has been made for the adult Djungarian hamster since daily melatonin injections given in long photoperiods (LD 16:8) elicit phase advances and extend the duration of α but only in those individuals which also respond by gonadal regression, moult and body-weight loss. Therefore, melatonin mimics short-day-like adjustments[22]. It has been pointed out[35] that the restriction of melatonin sensitivity to a narrow gate in the pacemaker system means that endogenous melatonin secretion will be present or absent at this phase depending on season and photoperiod. For seasonal breeders in long scotoperiods in winter, coincidence would occur and small daily phase shifts in gonadotrophin rhythms induced, resulting in changes to hormonal secretory patterns, gonadal status and reproductive viability. Since in nature it takes many weeks for gonadal status to change, it is not necessary for melatonin to elicit large, abrupt phase shifts. Gradual, small daily shifts in gonadotrophin rhythms would be adequate[35].

Primates and applied aspects

While the administration of exogenous melatonin clearly affects the circadian organization of Long-Evans hooded rats, and results of melatonin administration on the circadian system of hamsters are starting to emerge in the literature, the situation in primates remains uncertain. One methodological difference between the rodent and primate studies, which may or may not be relevant, is that in the former melatonin is administered by injection while in the latter oral administration is the preferred route.

Daily oral administration of melatonin (150 µg), 2.5 h before dark onset, to isolated pairs of adult saddle back tamarins maintained in LD 12:12, decreased locomotor activity late in the photophase and increased activity early in the photophase, but did not phase shift the activity pattern[20]. In a second experiment, melatonin was administered daily at the same clock time for 15 days under a regime where the LD cycle was advanced by 1 h per day until an LD reversal had been completed. Melatonin failed to prevent entrainment to the LD cycle. The authors concluded that melatonin may not have the same importance in the circadian organization of primates as it has in the rat[20].

Two studies involving blind humans indicate that melatonin may affect the circadian system but in both cases the reports are preliminary and lack details. In a single-blind, placebo-controlled, cross-over design study[31], oral melatonin (5 mg) was compared to triazolam when given at the same clock time every day to two free-running blind subjects. Both compounds produced phase advances of the free-running endogenous melatonin rhythm. In one subject melatonin induced a 7.6-h phase advance over 28 days while triazolam had an equivocal effect over 30 days; in the second subject, triazolam induced a 6.9-h phase advance while melatonin only caused a 1.6-h advance. In a second study[2], a single blind subject with a severely disrupted and possibly free-running sleep-wake rhythm, responded to melatonin by improved synchronization of sleep onset, elimination of daytime naps and apparently by synchronization of the endogenous melatonin rhythm. These results augment the findings that oral melatonin successfully alleviates the effects of jet lag[1,7,9,32,33]. In the most detailed study[1], jet lag, incurred after eight eastward time zone changes, was ameliorated by melatonin ingestion and re-entrainment of the endogenous melatonin and cortisol rhythms was accelerated. Results from all of these studies encourage the notion that exogenous melatonin administration may act upon the human biological clock and the recent finding that hypothalamic melatonin receptors are concentrated in the human SCN[30] favour this interpretation.

It should be noted that there are two reports of negative effects of oral melatonin (5 mg) on the synchronization of the human sleep-wake cycle[41]. In the first study involving two subjects, two 6-h phase delay shifts (simulating westward flights) were implemented, with placebo taken after the first shift and melatonin after the second. While it was concluded that there was no difference in the rate of re-entrainment between melatonin and placebo

treatments, the data show that this was because the sleep wake cycle re-entrained within one day in both subjects and therefore the jet lag situation was not simulated. The re-entrainment rate of the rectal temperature rhythm by both melatonin and placebo administration was the same in one subject, while melatonin treatment induced faster entrainment in the second subject. From the data provided, the rate of re-entrainment of urinary potassium was unclear. The second study involved the fractional desynchronization procedure which consists of the daily lengthening of the LD zeitgeber period until approximately 29 h is reached. Melatonin was administered every evening at bedtime in an attempt to strengthen the zeitgeber and prevent internal desynchronization. Melatonin was unable to prevent internal desynchronization of the rectal temperature rhythm which broke away from the sleep-wake cycle to follow a shorter τ. In contrast, melatonin synchronized the rhythm in fatigue-alertness to the zeitgeber. Normally, without melatonin administration, fatigue-alertness breaks away from the zeitgeber in approximately half of the subjects[3,41]. The results of studies conducted in temporal isolation are therefore equivocal, but an important point is that melatonin was not given at the optimum time. From studies on rats and one other human study[4], it is clear that to be most effective, melatonin should be given several hours before subjective evening.

Alternative explanations

The main issue for debate is whether it is merely coincidence that the entrainment properties of melatonin in rats fit reasonably neatly with the recent evidence that hypothalamic melatonin receptors are concentrated in the SCN, the biological clock of the central nervous system. If this were found to be the case, the melatonin entrainment results would represent a pharmacological artefact rather than a physiological finding, and free-running locomotor rhythms merely represent a convenient bioassay for testing melatonin. In the context of the physiological versus pharmacological arena it is important to evaluate viable alternative explanations of the findings and in this context phylogeny and ontogeny become fundamental.

Phylogeny

Effects of exogenous melatonin may reflect activation of vestigial remnants of a pineal-circadian system important in ancestral species at an earlier stage in evolution (Underwood, this issue). Presumably, during evolution, with loss of the parietal foramen or changes to skull thickness and ossification, environmental lighting information became no longer available to the pineal body nor to CNS photoreceptors through the skull; lighting information to the pineal had to be obtained through an indirect route and concomitantly the SCN acquired the master pacemaker function. There is apparently little supportive evidence for this hypothesis. 'The pineal gland is clearly not the biological clock controlling rhythms of locomotor activity in teleost fish and lizards'[39], p. 70. Robust circadian activity rhythms are found in a species of alligator which has no discernible pineal gland[19] and in lizards various results of Px are found which include arrhythmicity, changes to τ and no effect on circadian rhythmicity (see Underwood, this issue). There is little to suggest that, as a general rule, the pineal is a biological clock in reptiles although it clearly is a circadian pacemaker in certain species of lizard. This statement could simply reflect the lack of diversity of reptile species studied, but this is unlikely because parallel findings occur in birds where a larger number of species have been examined. Indeed, some nocturnal species of birds are characterized by having an atrophied pineal body[23]. Not only are there different results from Px between species of birds (see Armstrong[6] for review) but different results may occur within one species, the European starling[17]. To account for intra-species differences to Px it was proposed that variations in strength of internal coupling between self-sustained oscillators would dictate the effect of Px on the circadian system[17], and that melatonin would act as an internal synchronizing agent directly affecting one or more of the self-sustained oscillators[18] (Underwood, this issue). Removal of melatonin by Px would result in behavioural arrhythmicity if coupling were weak, or have no effect if coupling were strong. Following this same logic, negative findings from Px studies on rodents do not necessarily indicate lack of pineal involvement in the mammalian circadian system, where SCN oscillatory coupling may be strong. Nevertheless, there is no evidence for a mammalian ancestral archetype in which the pineal was a circadian pacemaker and from which modern species descended with loss of pineal pacemaker function.

Ontogeny

The effects of melatonin administration on the circadian system of adult rats may be due to stimulation of the rudiments of an ontogenetic system normally redundant in adults, having declined during development, but which is capable of being activated when melatonin is administered in supra-physiological amounts. It has been suggested that melatonin could be an important source of entraining information for the fetal biological clock[30], since: 1) melatonin binding sites are found in the SCN of the human and Djungarian hamster foetus[30,40]; 2) in rats the SCN oscillates before being innervated by the retinohypothalamic tract and the maternal circadian system entrains the fetal SCN to the external LD cycle[28]; 3) daily melatonin injections to pregnant, SCN-lesioned female hamsters set the subsequent phase of the pups' circadian activity rhythm and therefore presumably entrain the fetal rhythm[15]. However, two observations

must be accounted for within the ontogenetic explanation. First, although maternal Px alters the metabolic rhythm in the rat fetal SCN, it does not prevent synchrony of the pups' circadian rhythms in the same way that SCN lesions do [29]. Second, entrainment of hamster pups to melatonin injections is not limited to the narrow gate of approximately CT 10 and 11 as found in adult rats [10]. Nevertheless, the ontogenetic explanation has merit and needs to be explored further.

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