Melatonin administration can entrain the free-running circadian system of blind subjects

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Abstract

Although melatonin treatment has been shown to phase shift human circadian rhythms, it still remains ambiguous as to whether exogenous melatonin can entrain a free-running circadian system. We have studied seven blind male subjects with no light perception who exhibited free-running urinary 6-sulphatoxymelatonin (aMT6s) and cortisol rhythms.

In a single-blind design, five subjects received placebo or 5 mg melatonin p.o. daily at 2100 h for a full circadian cycle (35-71 days). The remaining two subjects also received melatonin (35-62 days) but not placebo. Urinary aMT6s and cortisol (n=7) and core body temperature (n=1) were used as phase markers to assess the effects of melatonin on the circadian system.

During melatonin treatment, four of the seven free-running subjects exhibited a shortening of their cortisol circadian period (tau). Three of these had taus which were statistically indistinguishable from entrainment. In contrast, the remaining three subjects continued to free-run during the melatonin treatment at a similar tau as prior to and following treatment. The efficacy of melatonin to entrain the free-running cortisol rhythms appeared to be dependent on the circadian phase at which the melatonin treatment commenced.

These results show for the first time that daily melatonin administration can entrain free-running circadian rhythms in some blind subjects assessed using reliable physiological markers of the circadian system.

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Introduction

The ability of the pineal hormone melatonin (N-acetyl 5-methoxytryptamine) to phase shift the circadian system has been extensively investigated in humans. The first demonstration of such an effect was by Arendt and co-workers (1985*a*) who showed a phase advance (1-3 h) of the endogenous plasma melatonin rhythm in 5 out of 11 subjects following 2 mg melatonin administered daily at 1700 h for 3 weeks. Subsequently, human phase response curves (PRC) to melatonin have been constructed using various melatonin doses and regimes (Lewy *et al.* 1992, Zaidan *et al.* 1994, Lewy & Sack 1997, Middleton *et al.* 1997, Lewy *et al.* 1998). Generally, the studies concur, that when melatonin is administered in the late subjective day/early subjective night, phase advances are induced and when given in the late subjective night/early subjective day, phase delays occur. However, in humans, it is difficult to achieve the steady-state free-running circadian rhythms which are a prerequisite to assess phase shifts and to construct a PRC. Unfortunately, the variety of approaches, the different physiological markers and definitions of phase used to construct PRCs have led to some ambiguity as to its exact shape.

Although melatonin has been shown to phase shift the human circadian system, there have been few reports of entrainment by melatonin in humans. Evidence of entrainment of core body temperature (CBT), hormonal and sleep-wake rhythms by melatonin treatment has been shown in some sighted (Middleton *et al.* 1997, Hashimoto *et al.* 1998, Hayakawa *et al.* 1998) and blind adults (Arendt *et al.* 1988) with free-running rhythms. Sack and colleagues (Sack *et al.* 1987, 1990, 1991) have shown cumulative phase advances of the endogenous melatonin and cortisol rhythms of up to 8.4 h in free-running blind males following daily melatonin administration (0.5 mg and 5 mg) at 2200 h or bedtime for up to 21 days. The claim that a free-running blind man exhibited entrainment following several years of self-medication of melatonin (Sack *et al.* 1990, 1991) has recently been questioned (Sack & Lewy 1997).

Despite the lack of evidence for consistent entrainment of physiological rhythms in humans, several studies have shown stabilisation of the sleep-wake rhythm and improved sleep in blind adults during melatonin treatment (Arendt *et al.* 1988, Aldhous & Arendt 1991, Tzischinsky *et al.* 1992, Arendt *et al.* 1997). Similar improvements have been shown in sighted free-running subjects (Kato *et al.* 1998, Siebler *et al.* 1998) but not in all cases (Okawa *et al.* 1998). However, in the blind only one of these studies assessed the effect of melatonin administration on a circadian phase marker (Folkard *et al.* 1990). In addition, the strong homeostatic influence on sleep (Borbély 1982) prevents the assumption that other circadian rhythms were similarly stabilised.

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Table 1 Subject details

Subject no.	Age	Diagnosis	No. of intact eyes	Onset of visual loss	Rapidity of visual loss	Melatonin start date	No. of days melatonin
S18	45	Retinopathy of prematurity	0	Birth	–	25 Mar	35
S45	43	Ocular trauma	0	1986	Days	2 Nov	48
S51	60	Retinoblastoma	1	Birth	Years	21 Nov	60
S17	47	Microphthalmia	2	1965	Years	5 Feb	71
S31	33	Retinoblastoma	0	1965	Years	3 Jun	35
S26	46	Buphthalmos	1	Birth	–	28 Jul	62
S62	38	Retinoblastoma	0	1961	Years	13 Oct	35

–, not applicable.

The aims of our study were firstly to evaluate the repeatability of tau measurements in field studies of free-running blind subjects. Secondly, to assess the ability of exogenous melatonin to entrain the circadian system of free-running blind subjects. The protocol enables the effect of melatonin to be assessed during a stable, long-term free-run without any influence of the light-dark cycle, the strongest entrainment signal in humans.

Materials and Methods

Subjects

Seven male subjects were studied (Table 1). The study was approved by the University of Surrey Advisory Committee on Ethics and the subjects gave informed consent. Subjects were instructed not to take any medications that could affect melatonin and/or the sleep-wake cycle and urine tests for drugs of abuse prior to, during and post-melatonin treatment were all negative.

Five of the subjects had been previously shown to have free-running 6-sulphatoxymelatonin (aMT6s) rhythms (Lockley *et al.* 1997). The two remaining subjects (S51, S62) were confirmed as having a free-running aMT6s rhythm prior to commencing the melatonin treatment

Parameters measured

Subjects were assessed for 12-27 weeks. At 1-2 weekly intervals, subjects collected sequential 4-hourly (8-hourly overnight) urine samples for 24 (n=1) or 48 h (n=6) for the assessment of aMT6s, the major urinary metabolite of melatonin, and urinary cortisol. Both rhythms are considered reliable markers of circadian phase and have been shown to run in parallel in free-running blind subjects in both laboratory (Sack *et al.* 1992) and field (Skene *et al.* 1999) conditions. During melatonin treatment periods, cortisol was used as the marker of circadian phase. During the urine sampling, one subject (S45) also measured CBT continuously via a rectal temperature probe.

Treatment

In a single blind design, five of the seven subjects were treated daily for one full circadian cycle with 5 mg melatonin p.o. (Penn Pharmaceuticals, Tredegar, UK) or placebo (identical lactose-filled gelatin capsule) at 2100 h. The remaining two subjects (S26, S62) also received melatonin for a full circadian cycle but these data were compared with a no-treatment baseline. Table 1 shows the duration and time of year of the melatonin treatment.

Rhythm analysis

The aMT6s (ng/h), cortisol (nmol/h) and CBT rhythms were subjected to cosinor analysis (Nelson *et al.* 1979) to determine the acrophase times of the rhythms. Acrophase times (phi (φ)) were accepted if the probability of fit (p) was <0.05 for aMT6s and CBT and <0.20 for cortisol. To assess the period of the aMT6s, cortisol and CBT rhythm, acrophases were fitted with best-fit regression lines (tau (τ)=24+slope) (Lockley *et al.* 1997, Skene *et al.* 1999) during the pre-melatonin treatment period, the melatonin treatment and the post-melatonin period. The mid-point of the upward and downward mean crossing times (Shanahan *et al.* 1999) were also subjected to regression analysis. The parameters were considered free-running if the period of the rhythm was different to 2400 h and the 95% confidence limits of the regression line did not encompass 0 (i.e. 2400 h) and entrained if the period of the rhythm was close to 2400 h and the 95% limits did cross 0. Placebo and no-treatment data were analysed together. The overall tau was taken from the longest sequence of urine data without melatonin treatment.

Assays

The urinary aMT6s rhythm was measured by radio-immunoassay (RIA) (Arendt *et al.* 1985*b*, Aldhous & Arendt

Table 2 Tau analysis for urinary aMT6s and cortisol rhythms before, during and after treatment with melatonin in subjects who were a) not entrained by melatonin and b) entrained by melatonin

	Pre-melatonin		During melatonin	Post-melatonin	
	aMT6s tau (h) (± 95% limits)	Cortisol tau (h) (± 95% limits)	Cortisol tau (h) (± 95% limits)	aMT6s tau (h) (± 95% limits)	Cortisol tau (h) (± 95% limits)
a) not entrained					
S18	–	–	24.70±0.56	24.72±0.84	24.96±0.61
S45	24.52±0.34	24.87±0.21	25.22±2.59	24.91±0.20	24.97±0.11
S51	24.49±0.12	24.53±0.09	24.52±2.24	–	–
b) entrained					
S17	–	–	24.00±0.06	24.27±0.08	24.30±0.06
S31	24.57±0.24	24.57±0.23	24.03±0.20	–	–
S26	24.43	24.45	24.05±0.80	24.28	–
S62	24.81±0.13	24.80±0.20	–	24.75±0.08	24.91

–, not measured.

1988) with reagents obtained from Stockgrand Ltd (University of Surrey, UK).The limit of sensitivity (LOS) for the assay was 0.5 ng/ml. The interassay coefficients of variation (CV) were 19.0% at 3.8 ng/ml (n=26), 15.4% at 20.1 ng/ml (n=26) and 13.3% at 41.7 ng/ml (n=25).

Urinary cortisol was extracted with dichloromethane and measured by RIA (Riad-Fahmy *et al*. 1979) using an antiserum raised in sheep (Scottish Antiserum Production Unit, UK) and an iodinated radiolabel (Amersham International, UK). The LOS for the assay was 5.5 nmol/l. The interassay CV was 14.9% at 49 nmol/l (n=22), 11.2% at 185 nmol/l (n=20) and 15.3% at 861 nmol/l (n=20).

Results

The mid-point of the crossing times correlated well with the acrophase times for both the aMT6s (r=0.93) and cortisol (r=0.94) rhythms. Taus derived from midpoint crossing times and the acrophase times also correlated well (r=0.96 and 0.94 for aMT6s and cortisol, respectively). However for clarity, only taus derived from analyses of the acrophase times are quoted.

Repeatability of tau

The aMT6s (Lockley *et al*. 1997) and cortisol (Skene *et al.* 1999) rhythms of 5 subjects were assessed in a previous study using identical methodology. There was a good correlation between the taus measured in the two separate studies for both aMT6s (r=0.96, n=5) and cortisol (r=0.73, n=5).

aMT6s and cortisol output

The subjects' endogenous aMT6s output (mean±S.D.) ranged from 9.1±6.8 to 22.0±4.3 μg/24 h and was within the range for sighted subjects (mean ± 2 S.D. = 9.5 ± 16.6 μg/24 h, n=517; English & Arendt, unpublished results). It increased on average 169-fold (range 67 to 256-fold) during treatment which was again similar to comparable sighted subjects (mean increase of 198-fold, range 92 to 392-fold; Middleton *et al*. 1997). These data confirmed the subjects' compliance with the treatment order. During melatonin treatment, urinary aMT6s levels never fell to within the endogenous aMT6s range. There was no significant effect of melatonin treatment on the subjects' endogenous mean 24 h cortisol output. There were no significant differences in the subjects' aMT6s and cortisol 24 h output between the previous studies (Lockley *et al*. 1997, Skene *et al*. 1999) and the current one (n=5, P>0.05).

Effects of melatonin on the circadian system

Table 2 shows the aMT6s taus before and/or after the melatonin treatment and the cortisol taus throughout the study for all subjects. Overall, the circadian system, as assessed from the cortisol rhythms, did not appear to change during the melatonin treatment in three subjects (S18, S45, S51) compared with before and/or after the treatment (Table 2a). Thus these subjects did not appear to be entrained by the melatonin treatment (Fig. 1). Although they were clearly free-running, two subjects (S45 and S51) had large 95% confidence limits associated with the cortisol taus during their melatonin treatment (Table 2a) due to the long study duration and low frequency of sampling.

Similarly during melatonin treatment, CBT data for S45 showed a free-running tau with large 95% confidence limits (24.86±2.32 h). However, the tau was very similar to the pre- and post-melatonin treatment measurements (24.90±0.10 h and 24.88±0.20 h, respectively).

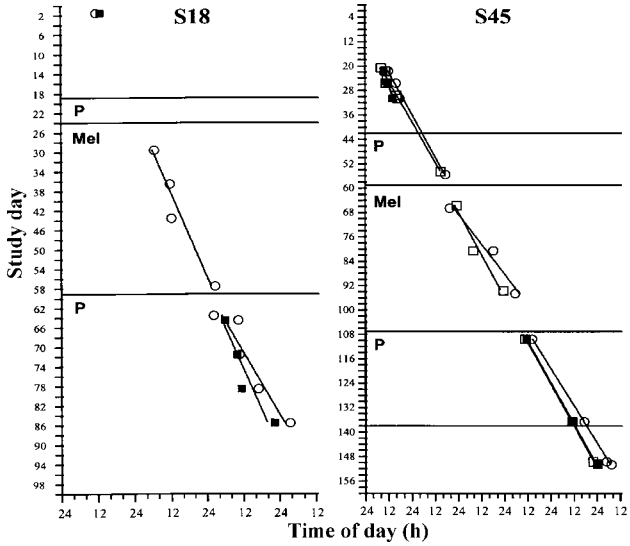

Figure 1 Acrophase times of the circadian phase markers for two subjects who did not entrain during melatonin treatment (S18, S45). Study days are shown on the vertical axis and the time of day is shown as a continuous sequence along the horizontal axis. Best-fit regression lines during each treatment are plotted for the aMT6s (■), cortisol (❍) and CBT (❑) data. The calculated taus are shown in Table 2. Duration of the placebo (P) and melatonin (Mel) is shown by the horizontal lines.

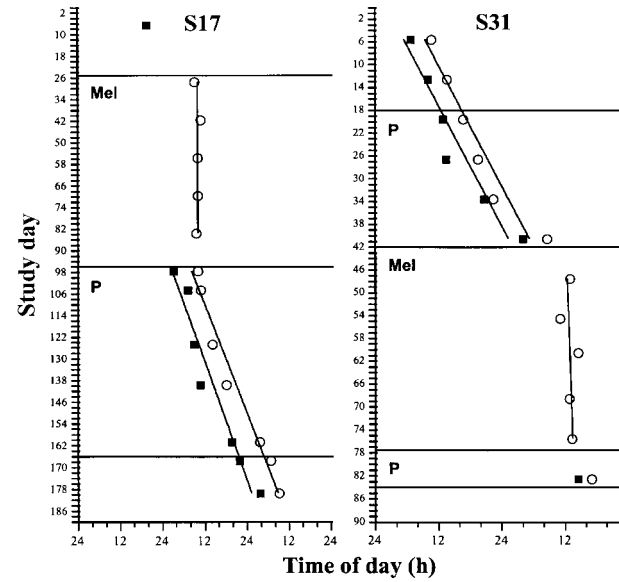

Figure 2 Acrophase times of the circadian phase markers for two subjects who entrained during melatonin treatment (S17, S31). Other details as in Fig. 1.

Three (S17, S31, S26) of the remaining four subjects exhibited a shortened cortisol tau which was statistically indistinguishable from entrainment (Table 2b and Fig. 2). For these subjects, the mean (±S.D.) cortisol acrophase times during melatonin treatment were 9.9±0.7 h (S17), 12.9±1.2 h (S31) and 11.5±1.5 h (S26) which were within the range for normally entrained blind subjects (mean±2S.D.=9.9±3.6 h, n=20; Skene *et al.* 1999). In the remaining subject (S62), a tau could not be calculated during the melatonin treatment due to insufficient data points. The measured cortisol acrophase time (12.4 h) was consistent with a shortening of tau as the acrophase time, assuming a free-running cortisol rhythm, should have been 23.6 h. However, the lack of data points precludes a definitive assessment of melatonin's effects in this subject.

The circadian time (CT) at which melatonin administration commenced was calculated by extrapolation from the aMT6s tau (where melatonin onset time = CT14 and aMT6s acrophase time = CT21). The three subjects who showed entrainment began their melatonin treatment at CT11 (S17), CT12 (S26) and CT16 (S31). S62, with the advanced acrophase time, began melatonin treatment at CT11. The three subjects who did not entrain commenced their treatment at CT3 (S51), CT5 (S45) and CT19 (S18).

Discussion

This study demonstrates for the first time that daily administration of 5 mg melatonin can entrain the free-running circadian system of some blind subjects. The good degree of repeatability of the tau measurement between this and our previous studies allows any change in tau during the melatonin treatment to be considered significant.

Entrainment by melatonin has not been previously demonstrated in blind subjects. In the study by Sack *et al.* (1991), four out of five blind subjects showed phase advances in their melatonin (n=4) and cortisol rhythm (n=3) following melatonin treatment but entrainment was not observed. The reason for this is not clear but may have been due to the short duration of treatment (21 days). The other extensive study of melatonin's effect on phase in a blind man (Folkard *et al.* 1990) did not demonstrate any change in the CBT or cortisol rhythms during melatonin treatment.

The most recently published PRCs (Lewy & Sack 1997, Middleton *et al.* 1997, Lewy *et al.* 1998) show that melatonin administration between approximately CT6 and CT18 (equivalent to 1300-0100 h clock time in a normally entrained subject) induces a phase advance and that between approximately CT18 and CT6 phase delays are predicted. Thus, in the present study, all the subjects who were entrained by melatonin began their treatment in an advance portion of the PRC whereas the three individuals who were not entrained, began melatonin treatment in the delay portion of the PRC.

These findings suggest that entrainment may be dependent on the phase at which melatonin treatment commences. The

results are certainly intriguing and are consistent with studies of free-running sighted subjects in that entrainment only occurred when melatonin was started during an advance portion of the PRC and not when started during the delay phase (Middleton *et al.* 1997). To test this, further studies are underway to administer melatonin to the same subjects as reported here at the opposite circadian phase.

However, there may be some problems with this hypothesis. For example, why did the subjects in the present study not entrain once they reached the appropriate advance phase, as would be predicted from the PRC and animal experiments (Redman *et al.* 1983)? A similar lack of entrainment was observed in two sighted subjects by Middleton and colleagues (1997).

Another explanation for the subjects' lack of entrainment in our study may be that exogenous melatonin is affecting melatonin receptor sensitivity (Stetson *et al.* 1986, Gauer *et al.* 1993). In addition, beginning melatonin treatment in the delay part of the PRC may somehow compromise the ability of melatonin to entrain the circadian system by, for example, altering the PRC such that phase advances are no longer possible.

In the present study, it may be that the duration of melatonin treatment in the non-entrained subjects was not long enough to ensure a continued period of melatonin exposure within the advance portion of the PRC. It is also possible that some individuals are simply insensitive to melatonin (Zhdanova *et al.* 1995). There may be large individual differences in PRCs and some individuals may be insensitive to the constantly high circulating levels of melatonin during treatment.

From the one entrained subject (S17) who received melatonin before placebo, there appeared to be no 'after-effect' of melatonin on circadian periodicity. S17 free-ran after stopping melatonin treatment with a tau (aMT6s, 24.27±0.08 h) (Fig. 2) comparable to that measured in a previous study (24.34±0.10 h; Lockley *et al.* 1997).

Apart from the circadian phase of treatment onset, no other measured parameter was consistently different between subjects who did and those who did not entrain with melatonin. There were no significant differences between the age of the subjects, the duration of treatment, the time of year of treatment, their endogenous 24 h aMT6s production, their aMT6s concentrations following melatonin treatment or their taus.

An alternative hypothesis to explain these results would be that all of the subjects had shortened taus during melatonin treatment. It could be argued that the acrophases at the end of the melatonin treatment for S18 (day 57), S45 (day 94) and S51 (day 165, not shown) were advanced by 24 h rather than plotted free-running as shown. This alternative hypothesis requires that the melatonin treatment induced net phase advances in all three subjects which were greater than their endogenous delays thus making their rhythms go 'backwards'. This is unlikely given the sinusoidal shape of the PRC as, at the point where the induced advance equals the endogenous delay, the rhythms would be expected to stabilise. More frequent phase assessment in future studies will prevent this ambiguity.

In summary, the present study shows the first demonstration of entrainment of free-running blind subjects by melatonin treatment assessed using reliable physiological markers of the circadian system.

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