Melatonin in mice: rhythms, response to light, adrenergic stimulation, and metabolism

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Melatonin in mice: rhythms, response to light, adrenergic stimulation, and metabolism. Am J Physiol Regulatory Integrative Comp Physiol 282: R358–R365, 2002; 10.1152/ajpregu.00360.2001.—There has been relatively little research conducted on pineal melatonin production in laboratory mice, in part, due to the lack of appropriate assays. We studied the pineal and plasma rhythm, response to light, adrenergic stimulation, and metabolism of melatonin in CBA mice. With the use of a sensitive and specific melatonin RIA, melatonin was detected in the pineal glands at all times of the day >21 fmol/gland in CBA mice but not in C57Bl mice. Both plasma and pineal melatonin levels peaked 2 h before dawn in a 12:12-h light-dark photoperiod (162 ± 31 pM and 1,804 ± 514 fmol/gland, respectively). A brief light pulse (200 lx/15 min), 2 h before lights on, suppressed both plasma and pineal melatonin to near basal levels within 30 min. Exposure to light pulses 4 h after lights off or 2 h before lights on resulted in delays and advances, respectively, in the early morning decline of plasma and pineal melatonin on the next cycle. Administration of the β-adrenergic agonist isoproterenol (20 mg/kg) 2 and 4 h after lights on in the morning resulted in a fivefold increase in plasma and pineal melatonin 2.5 to 3 h after the first injection. In the mouse, unlike the rat, melatonin was shown to be metabolized almost exclusively to 6-glucuronylmelatonin rather than 6-sulphatoxymelatonin. These studies have shown that the appropriate methodological tools are now available for studying melatonin rhythms in mice.

pineal gland; circadian rhythm; phase shift; 6-glucuronylmelatonin; 6-sulphatoxymelatonin

DESPITE THEIR USEFULNESS IN many diverse areas of biomedical research, there has been surprisingly little systematic research conducted on the production of melatonin by the pineal gland in laboratory mice. The most likely reasons for this situation are the small amount of blood that can be obtained from mice, their small pineal glands, and insensitive melatonin assays. Nevertheless, it has always been presumed that mice, like all other mammals, would synthesize melatonin during darkness. This assumption was challenged in 1986 when it was shown that C57Bl mice had <10 pg melatonin per pineal gland at various times during the day and night (8). Subsequently, it was shown that only two of the commonly used mouse strains, C3H and CBA, had high-amplitude melatonin rhythms with peak pineal melatonin content occurring 2 h before lights on (13). Other commonly used mouse strains (BALB/c, DBA/2, 129/Sv, AKR/J, and CF-1) had undetectable melatonin (<20 pg/gland) at 2, 6, and 10 h after darkness onset.

Melatonin is produced in the pineal gland by N-acetylation of serotonin by N-acetyltransferase (NAT) followed by O-methylation by hydroxindole-O-methyltransferase (HIOMT). NAT is the rate-limiting enzyme in the synthesis of melatonin and is under β-adrenergic control from the superior cervical ganglion. When the possible causes of the impaired melatonin production in mice were examined, it was found that C57Bl, AKR/J, and BALB/c mice had neither NAT nor HIOMT activity in the pineal gland, whereas another strain (NZB/BLNJ) had NAT but no HIOMT activity (7). The lack of NAT activity in the pineal gland was later confirmed in BALB/c mice (34). In the case of C57Bl6J mice, it was found that a point mutation in the NAT gene results in a truncated protein that has little or no enzyme activity (30), whereas different mutations are present in BALB/c and 129/Sv strains (30). To date, there have been no reports on the nature of the HIOMT mutations.

The aim of the present study was to evaluate pineal melatonin synthesis, secretion, metabolism, and the response to light in CBA, C57Bl, and BALB/c mice using a variety of approaches. Of particular interest was the possibility of establishing a method to monitor melatonin metabolite excretion rhythms in a similar way to that conducted in laboratory rats (16).

MATERIALS AND METHODS

Adult male CBA, C57Bl, CBAxC57Bl (F1), and BALB/c mice were obtained from the University of Adelaide Central Animal house, and wild derived mice (32) were obtained from the Animal Resources Centre (Canning Vale, Western Australia) where they had been maintained since birth on a 12:12-h light-dark photoperiod. On arrival in our facility, they continued to be on the 12:12-h light-dark photoperiod.
(lights off 1900, on 0700) and were fed standard laboratory mouse food ad libitum.

**Pineal and plasma melatonin rhythm studies.** Male CBA and C57Bl mice were killed by decapitation at 2-h intervals in dim red light, starting at 1900 and ending at 0700. Trunk blood was collected into heparinized tubes, and plasma was harvested by centrifugation and stored frozen before assay by RIA. Skull caps with attached pineal glands were cut away and frozen in 1 ml phosphate buffer containing 0.5% BSA (PBS/BSA). Before assay, pineals were removed together with the attached dura, homogenized in the buffer, and assayed for melatonin by RIA.

**Isoproterenol stimulation.** Groups of five CBA mice were injected with isoproterenol (20 mg/kg sc; Sigma, St. Louis, MO) or saline (CBA only) at 0900 and 1100, killed at 1130, and pineals and blood were collected as described.

Effect of light on pineal and plasma melatonin. Male CBA mice entrained to a 12:12-h light-dark photoperiod were killed at 0500, 0600, and 0700 (n = 14, 10, and 10, respectively). An additional group of five animals was exposed to 200 lx light for 15 min commencing at 0500 and killed at 0530. Pineal glands and blood were collected as previously described.

In two separate experiments, the effects of a 200 lx/15 min light pulse on the timing of the morning decline in pineal melatonin content and plasma melatonin concentration were determined. Male CBA mice entrained to a 12:12-h light-dark photoperiod were exposed to light pulses at either 2300 or 0500 or were left in darkness. The lights remained off for the remainder of the experiment, and groups of five animals were killed the next morning at 0500, 0600, and 0700. Pineal glands and blood were collected for melatonin analysis as above.

**Urine collections.** Male CBA and C57Bl mice (n = 5) were acclimatized in metabolism cages for several days and fed a liquid diet (Osmolite HN, Abbot Laboratories) as previously reported for rats (16). Urine was collected at 2-h intervals from 1900 to 0900 for two nights and for one night of continuous light. The mice appeared to tolerate the liquid diet, and urine volumes in excess of 200 μl/2 h were regularly obtained overnight, with the urine flow decreasing slightly before the expected time of lights on.

To investigate the nature of the metabolism and possible metabolites of melatonin in the mouse, five CBA and five C57Bl mice were housed in metabolism cages, placed on a liquid diet, and urine was collected at 2-h intervals. At 1600 (3 h before expected darkness), mice were injected subcutaneously with 100 μg melatonin, and the lights were kept illuminated. Samples (20 μl) were assayed for 6-sulphatoxymelatonin by RIA.

**Assays.** Melatonin was assayed in plasma from individual mice (50–100 μl) by RIA using reagents obtained from Buhlmann Laboratories (Allschwil, Switzerland). This assay uses the G280 antibody developed in our laboratory (18). Samples diluted to 1 ml with PBS/BSA were added to C18 reverse-phase columns, and melatonin was eluted according to the instructions accompanying the assay kit. Standards were reconstituted in PBS/BSA and were also extracted to ensure that both standards and samples were treated identically. Sensitivity of the assay ranged from 21 to 43 pM depending on the volume of sample used. Pineal gland melatonin was assayed in 100 μl of homogenate by direct RIA using the Buhlmann reagents. The sensitivity was 21 fmol/gland. Urinary melatonin was assayed by direct RIA of 20 μl of urine. There has been no formal attempt to prove that the immunoassay reactivity detected in the mouse plasma is authentic melatonin due to the very low amounts of sample available and the low levels detected. Nevertheless, the G280 antibody used has been well characterized in terms of specificity with a large number of synthetic indoles, and it has low cross-reactivity with all naturally occurring indoles (15, 18).

The urinary metabolite 6-sulphatoxymelatonin was assayed in urine by direct RIA as previously reported for rats (1, 17) using reagents obtained from Stockgrand, Guildford, Surrey, UK.

**Chromatography.** To investigate the metabolism of melatonin, CBA, BALB/c, and wild-derived mice and a Wistar albino rat were injected intraperitoneally with 4 mg (mouse) or 40 mg melatonin (rat), and urine was collected overnight. For chromatography, 5 μl of undiluted urine were applied to a silica gel thin-layer plate along with standard solutions of melatonin, 6-hydroxymelatonin, 6-sulphatoxymelatonin, and 6-glucuronoylmelatonin. The plates were developed in butanol: acetic acid:water (4:1:1), and indoles were detected by spraying with Ehrlich’s reagent (p-dimethylaninobenzaldehyde). Melatonin and 6-hydroxymelatonin were obtained from Sigma. 6-Sulphatoxymelatonin was synthesized using the method of Fellenberg et al. (9). 6-Glucuronoylmelatonin was synthesized enzymatically using a procedure originally designed for steroid glucuronolysis (36).

The reaction was stopped by freezing the mixture. Thin-layer chromatography revealed two major indole-positive spots, one corresponding to the starting material and another consistent with 6-glucuronoylmelatonin (22). Aliquots of the reaction mixture (0.5 ml) were applied to a C18 Sep-Pak cartridge (Waters), and the presumptive 6-glucuronoylmelatonin was eluted with 40% methanol (11). The material has not been crystallized or subjected to any further chemical analysis at this stage.

**Statistics.** The pineal and plasma melatonin rhythm data for CBA and C57Bl mice were analyzed by ANOVA, and differences between times at night were tested by post hoc Student-Newman-Keuls test. Significance was set at P < 0.05. All studies were conducted according to the guidelines set out by the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee of Adelaide University.

**RESULTS**

**Pineal and plasma melatonin rhythms.** Melatonin was detected in the pineal glands of CBA mice at all times from 1900 to 0700, increasing from 0100, peaking at 0500, and decreasing eightfold by 0700 (Fig. 1). The changes in melatonin content with time were significant (ANOVA; F = 5.6, P < 0.001). Post hoc analysis indicated that the 0500 levels (1,800 ± 514 fmol/gland) were higher than all other times except 0300. By contrast, melatonin content of the C57Bl mice never exceeded 55 fmol/gland (=12 pg/gland) at any time (Fig. 1). Nevertheless, ANOVA indicated that there was a significant effect of time (F = 3.5, P = 0.01) with the highest melatonin content (41 ± 4.5 fmol/gland).
occurring at 0500. The lowest value (20.2 ± 3.3 fmol/gland) occurred at 2100 [the values at 0500 were significantly higher than those at 2100 (P = 0.04) and 2300 (P = 0.047)].

Plasma melatonin levels in CBA mice reflected the patterns of pineal melatonin content with the highest plasma melatonin levels at 0300 and 0500, coinciding with the time of highest pineal content (Fig. 1). One plasma sample at 2100 returned an assay value of 200 pM that was more than six standard deviations outside the means of the 1900 and 2300 sample times. This animal had very low pineal melatonin content, and so data from this animal were excluded from further analysis. In C57Bl mice, plasma melatonin was <43 pM throughout the night (Fig. 1), although one sample at 0300 and two samples at 0500 returned assay results above 100 pM. These values were 6 to 12 standard deviations above the mean values for the strain. As in the case of the CBA plasma sample, unfortunately, there was insufficient sample available for a repeat analysis. The three C57Bl mice with “elevated” plasma melatonin had undetectable pineal melatonin content. Correlation analysis in the two strains indicated a significant relationship between pineal and plasma melatonin in CBA but not C57Bl mice.

Isoproterenol. Administration of isoproterenol (20 mg/kg) at 0900 and again at 1100 resulted in a robust and prolonged increase in pineal melatonin content and plasma melatonin levels 30 and 60 min after the last injection in CBA mice (Fig. 2). Saline administration to CBA mice caused no changes in pineal or plasma melatonin levels (pineal melatonin 79 ± 15 fmol/gland; plasma melatonin <30 pM). When the response of CBA and C57Bl mice to isoproterenol was compared 30 min after the last injection, the pineal melatonin levels were 413 ± 75 and <21 fmol/gland, respectively; plasma melatonin values were 63 ± 5 and <30 pM, respectively. No obvious acute ill effects were observed in the mice following isoproterenol administration.
**Light effects.** Exposure to light 2 h before dawn (0500) resulted in the suppression of pineal melatonin content and plasma melatonin levels to basal levels within 45 min of the start of light exposure (Fig. 3). These levels were indistinguishable from the usual 0700 values.

When mice were exposed to light at 2300 or 0500, kept in darkness, and then killed after 30 or 24 h, around the time of the expected melatonin peak (0500), there were significant effects on the timing of the

Figure shows the results for untreated animals (●; means ± SE; n = 5–10). Data from 5 animals that were light pulsed for 15 min at 0500 and killed at 0530 are shown with ●. Where no SE bar is shown, the line is obscured by the symbol.

**DISCUSSION**

The CBA mouse strain has a high-amplitude pineal melatonin rhythm with a peak 2 h before lights on (13, 33). The decrease during the 2 h before dawn is profound, with near basal levels measured at zeitgeber time 0 (ZT0; ZT12 is defined as lights off), and it is highly repeatable, indicating that it is likely to be under clock control. This is to our knowledge the first report of plasma melatonin measurement in CBA mice, and we have shown that the pineal melatonin content is highly correlated with plasma melatonin levels. Masana et al. (25) reported pineal and plasma melatonin rhythms in the related C3H strain and found that the highest plasma levels coincided with the lowest pineal melatonin content 2 h after dark onset. This relationship was not observed in animals kept under constant darkness conditions. It remains to be confirmed whether this finding is a special feature of the C3H strain. We also confirmed the greatly impaired melatonin synthesis in the commonly used C57Bl...
strain. The slight, but statistically significant, elevation in pineal melatonin content (but not plasma melatonin) in C57Bl mice toward light onset is difficult to explain considering that the mutation in NAT responsible for the low melatonin production has been shown to be due to a truncated nonfunctional acetyltransferase (30). Little is known about the nature of the HIOMT defect in mouse strains and whether the mutant enzyme retains some enzyme activity. It is possible that small amounts of N-acetylserotonin produced by other transferases are methylated by residual pineal HIOMT enzyme. The rhythm could then come from a low-amplitude rhythm in pineal HIOMT activity similar to the rhythm reported in rats (29). It should be pointed out that very low levels of pineal melatonin synthesis have also been reported elsewhere in C57Bl mice (33, 35).

Exposure to bright light at the time of maximal pineal melatonin content and plasma melatonin levels resulted in rapid suppression of melatonin production within 30 min. This is consistent with a previous report (12) that showed that pineal melatonin content in CBA mice decreased to basal levels after 5-min exposure to 100-lx light. The rapid decrease in plasma melatonin levels following light exposure as opposed to the gradual morning decline between 0500 and 0700 suggests that the decline may be a clock-controlled event. Although complete profiles were not obtained due to the large numbers of animals required, a light pulse in the early dark period clearly resulted in a small but significant delay of the morning decline in melatonin production and secretion, whereas a pulse 2 h before lights on resulted in what we interpret as an advance of more than 2 h. These results are consistent with light-induced phase shifts in activity rhythms induced by light pulses in this strain (Kennaway et al., unpublished results) and other strains (3–5), although the

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Fig. 4. Effect of a light pulse 4 h after lights off (zeitgeber time 16 (ZT16); A, B) or 2 h before dawn (ZT22; C, D) on pineal melatonin content (fmol/gland) and plasma melatonin concentration (pM) around the time of the expected melatonin peak the next subjective morning. Note that the mice were kept in darkness from the time of the pulses until they were killed. Data are means ± SE (n = 5). The untreated animals are indicated with ● and pulsed animals with ○. *Pulsed and unpulsed groups were significantly different (P < 0.05) at that time of day. Where no SE bar is shown, the line is obscured by the symbol.

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Fig. 5. Urinary 6-sulphatoxymelatonin (aMT.6S) excretion rate in CBA and C57Bl mice in a 12:12-h light-dark photoperiod and over 1 night of constant light. Data are means ± SE (n = 5) with CBA mice shown as ■ and C57Bl mice as ○. The dark period is indicated by black horizontal bars at top.
magnitude of the shifts is smaller. This may be due to the use of the Aschoff Type II protocol (2) rather than the application of pulses after a long period of constant darkness.

The apparently large phase advance in the melatonin rhythm within one cycle following light exposure at 0500 was a little unexpected because in rats, it can take up to 4 days under similar circumstances for advances of the pineal NAT rhythm to become apparent (14). A possible explanation for this difference is the species difference in the endogenous period, which for rats free running in constant darkness is greater than 24 h and for mice less than 24 h [CBA mice in our laboratory have a free-running rhythm of wheel running of \( \sim 23.4 \) h (Kennaway et al., unpublished results)]. Thus, in rats, morning light pulses before producing a net advance must first counteract the natural tendency to delay, whereas in mice, morning pulses assist the natural tendency for rhythms to advance.

Recent in vitro studies by von Gall et al. (35) highlighted the similarities between rats and mice in the control systems responsible for the induction of NAT and synthesis of melatonin. We have shown that in vivo stimulation of the mouse pineal gland by the \( \beta \)-adrenergic agonist isoproterenol during the light period increased pineal melatonin content and melatonin secretion into blood in CBA but not C57Bl mice. In preliminary studies, we observed only small transient increases in melatonin production following a single injection of isoproterenol. By contrast, two injections, 2 h apart in the morning, provided reliable induction of melatonin synthesis, which is reminiscent of the requirements of Syrian hamsters for prolonged adrenergic stimulation to increase melatonin synthesis (31). Interestingly, both mice and hamsters have maximum melatonin production late in the dark period. The failure to detect increased melatonin production or synthesis in C57Bl mice in response to isoproterenol administration supports the hypothesis that the small amounts of melatonin detected in the pineals of some animals at night may be synthesized from \( N \)-acetylserytonin produced by a transferase enzyme outside the pineal gland that is not induced by \( \beta \)-adrenergic stimulation.

Melatonin produced by the pineal gland of mammals appears in the blood and is metabolized by the liver to 6-hydroxymelatonin, conjugated, and excreted into the urine (20). The nature of the conjugation products of melatonin was reported over 40 yr ago in rats to be 6-sulphatoxymelatonin and 6-glucuronoylmelatonin (20), and this was subsequently confirmed in humans (9, 22, 23, 37). With the development of a RIA for 6-sulphatoxymelatonin (1), it has been possible to monitor the excretion of 6-sulphatoxymelatonin into the urine of humans (10), rats (17), Djungarian hamsters (21), minks (26), and pigs (19). In all cases, the excretion rate of 6-sulphatoxymelatonin is rhythmic and can be used as an index of pineal gland rhythmicity (10). There is a clear advantage in such a procedure due to the noninvasive nature and simplicity of urine collection that reduces the number of animals needed for...
experiments. It was thus expected that mice would produce and excrete 6-sulphatoxymelatonin and that RIA procedures might be used to follow pineal rhythms in this species. It should, however, be pointed out that while Kopin et al. (20) used both rats and mice in their influential study, the nature of the melatonin metabolites in their mouse studies was not reported.

We have provided unequivocal evidence that mice do not excrete significant amounts of 6-sulphatoxymelatonin, presumably due to a lack of an appropriate sulphotransferase. Instead, melatonin is excreted as 6-glucuronylmelatonin with only very small amounts excreted unmetabolized. Our method of detection of metabolites involved both RIA and thin-layer chromatography. In the case of the RIA, the antibody has 0.5% cross-reactivity with 6-glucuronylmelatonin, and this may be the cause of the increase in 6-sulphatoxymelatonin immunoreactivity observed in CBA mice between 0600 and 1000 and following melatonin administration. In the case of the thin-layer chromatography identification of metabolites, because Ehrlich’s reagent only detects intact indole rings, we can neither exclude nor quantitate other nonindolic melatonin metabolites in the mouse. It is also important to point out that the inability to produce 6-sulphatoxymelatonin is not confined to laboratory mouse strains because the wild mouse strain also excreted only 6-glucuronylmelatonin.

The very low levels of unmetabolized melatonin excreted are in contradiction to a study by Perissin et al. (28), who not only reported melatonin in mouse urine but also found a more than fivefold increase in unmetabolized melatonin in urine at night. These researchers used a strain of mouse (BD2F1) that is a cross of two strains (DBA2 and C57Bl) both known to be melatonin deficient (13). A more recent study describing atypical plasma and pineal melatonin rhythms in this strain is remarkable for the extremely low levels of melatonin measured (24). We cannot provide an explanation for this discrepancy other than the possibility that the assay used was not specific for melatonin. Similar conclusions may be warranted in the case of the two recent publications that have challenged the idea that BALB/c, C57Bl, and AKR mice are melatonin deficient. In the first study, in each strain, melatonin was reported to be elevated for 15 min around ZT18; at all other times, the pineal melatonin content was very low (6). In the second study, melatonin was detected in BALB/c, C57Bl/6, and OF1 Swiss mice pineal glands, but the levels were very low and the pattern was very different from that observed for CBA and C3H mice (6).

Perspectives

The results of this study indicate that the CBA mouse strain has a robust melatonin rhythm that is under β-adrenergic control and that melatonin production can be suppressed by brief light pulses. There is also compelling preliminary evidence that light pulses presented at different times of the night phase shift melatonin rhythms. The need to kill animals for pineals or blood may restrict the usefulness of mice in studies of melatonin rhythmicity, although the knowledge that 6-glucuronylmelatonin is the major urinary melatonin metabolite provides an incentive to develop assays for this compound.

Apparently, all knockout and transgenic mouse lines are on genetic backgrounds that make the animals melatonin deficient (27). This is not acceptable for researchers studying circadian rhythms or reproduction. If strains of interest (i.e., knockout) are crossed with CBA mice, an isoproterenol stimulation test based on results presented in this study would provide a simple method of monitoring the inheritance of melatonin production capacity at least until the mutations in NAT and HIOMT are defined and PCR-based genotyping strategies are developed.

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