Melatonin in rat milk and the likelihood of its role in postnatal maternal entrainment of rhythms

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Rowe, Shawn A., and David J. Kennaway. Melatonin in rat milk and the likelihood of its role in postnatal maternal entrainment of rhythms. Am J Physiol Regulatory Integrative Comp Physiol 282: R797–R804, 2002; 10.1152/ajpregu.00228.2001.—The rhythm of melatonin in rat milk and the capacity of pups to synthesize and metabolize melatonin were studied. Melatonin was undetectable in milk in the light (<21 pM), but increased rapidly 2–4 h after dark to peak at 357 ± 66 pM at mid-dark. Oral or subcutaneous administration of melatonin to 5- and 10-day-old pups resulted in peak plasma melatonin levels 30 min after administration and rapid metabolism. Increases in pineal and plasma melatonin levels at night were detected at 5 and 6 days of age, respectively. Isoproterenol administration (2 μg/g body wt) at mid-light to day 10 pups increased plasma melatonin from 312 ± 40 pM to 1,298 ± 160 pM, whereas propranolol (2 μg/g body wt) suppressed nocturnal melatonin secretion from 1,270 ± 128 pM to 395 ± 66 pM. The rise of pineal and plasma melatonin in day 10 pups occurred 1 and 2 h after dark onset, respectively, preceding the onset in dams by 3 and 4 h, respectively. Propranolol administration to 2- and 5-day lactating dams inhibited plasma and milk melatonin at night but had no effect on their suckling pups. Transfer of melatonin via the milk is unlikely to provide an entraining signal for rat pups.

circadian; ontogeny; pineal; metabolism; 6-sulphatoxymelatonin

The Development of Circadian Rhythms and the Entrainment of These Rhythms to the Prevailing Photoperiod

The development of circadian rhythms and the entrainment of these rhythms to the prevailing photoperiod has been a topic of interest for many years, and yet there are still a number of unanswered questions. One such unresolved issue is the role of maternal rhythmicity in the entrainment of fetal and neonatal rhythms. Although the suprachiasmatic nucleus (SCN) is intrinsically rhythmic in the fetal rat (20), direct entrainment of the SCN by light does not appear to develop until the sixth postnatal day (4). It would appear that within this developmental period the mother establishes the appropriate phase relationship between the fetal SCN and the environment and reinforces this phase in the neonate until photoperiodic information can directly entrain the circadian rhythms (15).

Melatonin synthesized within the pineal gland is recognized as a likely hormonal signal for maternal-fetal entrainment. Several ontogenic studies (2, 14, 22–24) have suggested that rhythmic synthesis of melatonin in the pineal gland of rat pups does not appear until the second postnatal week. However, robust rhythms of melatonin in the maternal circulation are sustained throughout pregnancy, and placental transfer of melatonin to the fetus has been demonstrated in rodents, sheep, and primates (11, 13, 17, 27, 30, 35). Melatonin binding sites have been identified in the fetal SCN (34), and injections of melatonin into SCN-lesioned dams entrained the phase of the fetal SCN (3). Although melatonin may act as an entraining signal in late gestation, removal of the maternal pineal gland and thus circulating rhythmic melatonin did not abolish maternal entrainment of the fetal rat SCN (19). Therefore other hormonal or behavioral signals must also be involved in maternal-fetal circadian rhythm synchronization (28, 31).

Postnatal transfer of melatonin via the milk to the neonate has been proposed as a possible maternal entraining signal, although the evidence is only circumstantial. A rhythm of melatonin has been observed in human milk (10), but studies in other species are lacking. Indirect evidence for milk as a source of melatonin to the neonate has been provided from a study of the administration of exogenous melatonin to dams and suckling pups. Radiolabeled melatonin injected into lactating rat dams appeared in the stomach of suckling pups (18), and entrainment of circadian rhythmicity has been demonstrated with daily administration of melatonin to neonatal hamsters and rats (8, 25). These studies laid the framework for a possible mechanism through which melatonin transferred via milk may potentially act.

The present study was designed to directly address several outstanding questions relating to the hypothesis that melatonin transferred via milk may be an entraining signal to the suckling rat. There have been no studies on the content and rhythm of melatonin in rat milk. Therefore melatonin was quantitated in the milk of lactating rats, and the oral bioavailability and
clearance of melatonin in rat pups were determined to assess the likelihood of maternal-derived melatonin affecting the hormone levels in the neonate. In addition, the role of the neonatal pineal gland as a rhythmic source of melatonin during the early postnatal period was thoroughly reinvestigated using a sensitive and specific melatonin radioimmunoassay.

MATERIALS AND METHODS

Animals

Mature male and virgin female Wistar albino rats (body wt 220–250 g) were obtained from the Central Animal House facility (University of Adelaide). On arrival at the medical school facility, the animals remained on a 12:12-h light-dark photoperiod [lights on at 0700 denoted by zeitgeber time (ZT)0 with intensity 200 lx] at 25°C and were provided with pelleted rat chow and water ad libitum. Rats were paired, and females were checked for sperm each morning (denoted as day 0 of pregnancy). Males were withdrawn after 6 days. On the day of birth (22 days after sperm detection) litters were weighed and reduced to 10 pups.

Sampling Procedures

**Milk.** The procedure involved inducing light anesthesia with 3% halothane-oxygen and a subsequent injection of oxytocin (1 IU/100 g, Heriot Agret). Teats were softly manipulated and the milk was drawn into a series of 100-μl capillary tubes thereby generating samples of 500–600 μl from 6 teats. For the night samples, animals were anesthetized in complete darkness with the aid of infrared viewing equipment. Animals’ heads were covered with black plastic, and milking was conducted in dim red light. Milk was stored at −20°C until assays were performed.

**Pineal glands.** Dams and pups were decapitated in dim red light and individual pineal glands were immediately removed and stored frozen in 1 ml of radioimmunoassay buffer until homogenized for assay.

**Blood.** Trunk blood was collected into heparin-coated tubes and centrifuged. Individual plasma samples were obtained from 3-day-old or older pups. It was necessary to pool two blood samples from 1- and 2-day-old pups to obtain sufficient plasma for the assay.

**Urine.** Collection of urine was facilitated by our observation that 5- and 10-day-old pups do not appear to urinate unless licked in the urogenital area by the dam. When urine collections were required after melatonin administration, we were confident that there was no loss of sample through urination. After decapitation, urine leakage was prevented by the application of a pressure clip. After blood and pineal gland collection, the bladder was exposed, the urine was aspirated with a syringe, and the urine volume was estimated by weight.

**Melatonin in Milk**

Lactating rats (n = 5) on postnatal day 10 were separated from their pups and milked every 2 h over a 24-h period. In a separate experiment, 6 dams on either postnatal day 2 or 5 were briefly separated from their pups at mid-light and mid-dark and milk was obtained as above. Dams were used on only one occasion.

**Melatonin Clearance and Metabolism**

Pups (5 and 10 days old) were removed from their mothers and weighed, and melatonin [43 pmol (10 ng) in 10 μl of water] or vehicle was carefully and slowly introduced into the mouth of 5 pups from each litter using a 20-μl Gilson laboratory pipette. Any pups that showed evidence of leakage of melatonin solution from the mouth were excluded. At 30, 60, 120, and 240 min postadministration, groups of 5 animals were killed, and trunk blood was collected. A second group of 5-day-old pups received a subcutaneous injection of melatonin [43 pmol (10 ng) in 50 μl of saline] or vehicle and was killed at 30, 60, 120, and 240 min, and blood and urine were collected from 5 animals at each time point. Both experiments were performed between 1100 and 1600.

**Ontogeny of Melatonin Production**

To study the ontogeny of melatonin production, half of the rat pups from three litters were killed at mid-light and the remaining pups were killed at mid-dark from day 1 to day 8. Thus at each time point, there were 8–10 rats from three separate litters. Pineal glands and blood were obtained for melatonin assays. Owing to the low blood volumes of 1- and 2-day-old pups, blood plasma from two animals was pooled for assay; from postnatal day 3 onward, individual plasma samples were assayed.

**Adrenergic Regulation of Neonatal Melatonin Production**

On postnatal day 10, half of the pups from each of two litters (n = 8–10 pups) were injected subcutaneously with saline (50 μl) and the remainder were injected with isoproterenol (2 μg/g body wt; Sigma, St. Louis, MO). In a separate experiment, half of the pups from each of two litters (n = 8–10 pups) were injected with saline (50 μl), and the remainder were injected with propranolol (2 μg/g body wt; Sigma). Three hours postinjection (mid-light and mid-dark), pups were killed, blood was collected, and pineal glands were removed. For the night injections, pups were separated from their mothers with the use of infrared vision equipment, and with the aid of a dim red light source they were treated with the drugs.

**Timing of the Melatonin Onset**

Litters (n = 5) of 10-day-old pups (litter size, 9–10 pups) were killed, 1 per h from 2 h before (ZT10) until 6 h after (ZT18) lights out. During this time, the pups were left with their mothers. In a separate study, groups of 10-day lactating dams were killed at hourly intervals (4–5 per time point) between ZT10 and ZT18. Blood and pineal glands were collected at these times.

**Effect of Adrenergic Antagonism on Milk Melatonin and Maternal and Neonatal Plasma Melatonin**

Groups of four dams on postnatal days 2 and 5 were injected with saline vehicle or propranolol (10 mg/kg body wt) 2 and 4 h after the onset of darkness and were milked (as previously described) 2 h later. After collection of the milk, blood was collected via cardiac puncture. The pups belonging to these dams were killed at the same time, and plasma was assayed for melatonin. Because of the low blood volume of postnatal day 2 pups, plasma from two animals was pooled to yield 20 samples. For the postnatal day 5 pups, 40 individual samples were assayed.

**Melatonin Radioimmunoassays**

Melatonin was assayed by radioimmunoassay using iodinated melatonin (NEN Research Products), the Kennaway G280 melatonin antibody (26, 29), and an anti-sheep/goat second antibody.
Pineal glands and urine. Pineal gland melatonin was assayed directly in 100 μl of 1 ml of homogenate from rat pups aged <5 days and 100 μl of a 1:10 dilution of homogenate for animals aged ≥5 days. The detection limits of these assays were 8.6 and 86 fmol/gland, respectively. The interassay coefficient of variation was 12% at 15.5 fmol/tube. Intra-assay coefficients of variation were always <10%. Melatonin was directly assayed in 10 μl of urine with a detection limit of 86 pM.

Blood. Plasma (125 μl) and standards (1 ml; Buhlmann Laboratories, Basel, Switzerland) were extracted using C-18 reverse-phase extraction columns and were assayed as previously reported (29). After being reconstituted with buffer, 400-μl aliquots of standards and samples were assayed. The detection limit of the assays was 17.2 pM. The interassay coefficient of variation was 12% at 18.0 fmol/tube. Intra-assay coefficients of variation were always <10%.

Milk. An alternate extraction method was required for the milk samples (5) because the milk tended to block the C-18 reverse-phase columns. In brief, milk (50 μl) and standards (500 μl) were extracted with 2.5 ml of a 1:1 hexane-dichloromethane mixture. The solvent evaporated, and the sample was reconstituted in 400 μl of assay buffer. The sensitivity of the assays was 22 pM. All milk samples were analyzed in two assays. Intra-assay coefficients of variation were always <10%.

6-Sulphatoxymelatonin Radioimmunoassays

Urinary 6-sulphatoxymelatonin was assayed by radioimmunoassay (1). All samples were analyzed within a single assay with a detection limit of 3 fmol/tube. Displacement of 20, 50, and 80% of the radioligand was achieved with 8.54, 35.4, and 146.8 fmol/tube of melatonin. Intra-assay variation was always <10%.

Statistics

Data were analyzed using a general factorial ANOVA. Between-group differences were analyzed post hoc using multiple t-tests and significance levels were adjusted with the Bonferroni correction. Melatonin onset and offset times were determined using a one-way ANOVA and post hoc t-tests. For the day-night melatonin ontogeny study, individual nighttime data were tested against the pooled data of the previous and subsequent daytime (i.e., night 4 vs. day 4 and day 5). This method was chosen to compensate for the increasing basal values observed over the initial days of development (i.e., nighttime levels were higher than previous daytime levels simply because the values were measured 12 h later in development).

RESULTS

Melatonin in Milk

Milk was obtained on each sampling occasion for 24 h from 3 rats and for 18 h in the remaining 2 animals. Melatonin in milk was below the detection limit of the assay (22 pM) for most samples taken during the photophase and during the first 3 h of darkness. Melatonin concentration increased in the milk between 3 and 4 h after darkness, achieved peak levels of 200–550 pM, and decreased within the last hour of darkness (Fig. 1A). A significant midday and midnight difference in the melatonin concentration in milk was also observed from dams at earlier postnatal stages (Fig. 1, B and C).

Fig. 1. Two hourly milk melatonin concentrations (in pM, A) over a 24-h period in 5 individual 10-day lactating rats on a 12:12-h light-dark photoperiod (lights off at zeitgeber time (ZT)12). Data was plotted against the time recorded at the beginning of each milking for individual rats. Solid bar represents the dark period. Mid-light and mid-dark (means ± SE; n = 4–6 rats; B) milk melatonin concentration in 5-day lactating dams. Mid-light and mid-dark (means ± SE, n = 4–6 rats; C) milk melatonin concentration in 2-day lactating dams. For B and C, *P < 0.05, day vs. night.

Oral Melatonin Administration to Neonatal Rats

The oral administration of melatonin (43 pmol) raised the plasma melatonin concentration above that of vehicle-treated pups for up to 4 h at 5 days of age (Fig. 2A) and for 30 min at 10 days of age (Fig. 2B). To estimate the total amount of melatonin in the blood, a plasma volume of 10% body wt was assumed. Using this estimate, the maximum recorded elevation of plasma melatonin at 30 min in 5- and 10-day-old pups represented 4.9 and 4.2% of the total administered melatonin, respectively. The prolonged elevation of melatonin in the blood of 5-day-old animals represented 1.1 and 0.9% of the initial dose at 2 and 4 h postadministration, respectively.

Melatonin Clearance

Subcutaneous injection of melatonin (43 pmol) resulted in elevated plasma melatonin within 30 min in 5-day-old rat pups compared with saline-injected animals (Fig. 3A). At this time, 3.3% of the administered dose was in the circulation. A rapid decline in circulating melatonin was reflected by an initial elevation of melatonin in the urine of these animals (Fig. 3C) and...
slower but significant accumulation of the melatonin metabolite 6-sulphatoxymelatonin (Fig. 3B). Because the pups did not urinate over the duration of the sampling, the urinary melatonin and 6-sulphatoxymelatonin excretion reported is the accumulated amount of analyte in the urine up to the time of sampling. After 4 h, the ratio between the accumulated urinary melatonin and its metabolite was 1:20, and the total amount of urinary 6-sulphatoxymelatonin at 4 h accounted for ~20% of the administered dose of melatonin.

**Ontogeny**

Melatonin was detectable in the pineal gland of rats on the day after birth and increased steadily through development. The earliest significant difference between mid-dark and preceding and subsequent mid-light pineal melatonin levels was observed on the night of postnatal day 5 (Fig. 4A). Thereafter a day-night difference was evident at all ages studied. A highly significant correlation was evident between the melatonin content in the pineal gland and the estimated total plasma melatonin of rat pups at both mid-light and mid-dark over the first 8 postnatal days (Fig. 5).

**Adrenergic Regulation of Neonatal Melatonin**

Administration of the β-adrenergic agonist isoproterenol to 10-day-old rat pups during the light period significantly stimulated melatonin production in the pineal gland and resulted in elevated circulating melatonin concentrations equivalent to nighttime levels. By contrast, pineal melatonin production was inhibited during the dark period by the β-adrenergic antagonist propranolol, and this was reflected in reduced plasma melatonin levels in the pups similar to those found during the light period (Fig. 6).

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**Fig. 2.** Plasma melatonin concentration (in pM; means ± SE, n = 5 rats) of rat pups after oral administration of 10 μl of water (○) or 43 pmol of melatonin in 10 μl of water (■) to 5-day-old (A) and 10-day-old (B) pups at 30, 60, 120, and 240 min postadministration; *P < 0.01, water vs. melatonin.

**Fig. 3.** Plasma melatonin (A), urinary 6-sulphatoxymelatonin (B), and urinary melatonin (C) levels at 30, 60, 120, and 240 min after saline (50 μl sc, ○) or melatonin administration (43 pmol in 50 μl of saline, ■) to groups of 5-day-old rats (n = 5/time point). Plasma melatonin results are expressed as a concentration (pM, means ± SE), whereas the urinary 6-sulphatoxymelatonin and melatonin excretions are the accumulated amounts (in pmol) at the various times. *P < 0.01, saline vs. melatonin.
Onset of Nocturnal Rise

In 10-day lactating dams, the nocturnal rise in pineal gland and plasma melatonin occurred 4 and 6 h after the onset of darkness, respectively (Fig. 7B). The nocturnal pineal gland and plasma melatonin increase in the accompanying 10-day-old pups preceded the increase in the dams by 3 and 4 h, respectively (Fig. 7A).

Pharmacological Inhibition of Maternal Melatonin

Mid-dark plasma and milk melatonin levels were significantly lowered in dams treated with propranolol 2 and 4 h after onset of darkness compared with saline-injected dams. The circulating levels of melatonin at mid-dark in the 2- and 5-day-old suckling offspring were unaffected by the suppression of maternal melatonin (Fig. 8).

DISCUSSION

The present study has demonstrated for the first time a high-amplitude rhythm of melatonin in the milk

Fig. 4. Melatonin content in the pineal gland (fmol/gland; A) and melatonin concentration (pM; B) in the plasma at mid-light (open bars) and mid-dark (solid bars) over the first 8 postnatal days (means ± SE, n = 8–10 rats; *P < 0.005, mid-dark vs. surrounding mid-light).

Fig. 5. Correlation between pineal gland melatonin content and estimated total blood melatonin (concentration /BW/10) for all mid-day (○) and mid-night (■) samples from postnatal days 1-8 pups (daytime r = 0.75, P < 0.005; nighttime r = 0.89, P < 0.005).

Fig. 6. Pineal gland melatonin content (in pmol/gland, left) and plasma melatonin concentration (in pM, right) in 10-day-old rat pups at mid-light (open bars) 3 h after an injection of saline (Sal, 50 μl) or isoproterenol (Iso, 2 μg/g body wt), or mid-dark (solid bars) 3 h after an injection of saline or propranolol (Pro, 2 μg/g body wt). Each bar represents the mean ± SE; n = 8–10 rats; * P < 0.05, daytime saline vs. isoproterenol; **P < 0.05, nighttime saline vs. propranolol.

Fig. 7. Nocturnal onset of plasma melatonin concentration (in pM, □) and melatonin content in pineal glands (in pmol/gland, ■) in 10-day-old pups; n = 5 rats/time point from 5 litters (A), and 10-day lactating dams; n = 4–5 rats (B). Solid bar represents the dark period. *P < 0.05; **P < 0.05, time the values for pineal glands and plasma, respectively, first exceeded baseline levels.
of rats, and has shown that the pattern and concentration of melatonin in milk reflects circulating plasma melatonin levels. The similarity in the plasma and milk melatonin concentration was predicted on the basis of indirect studies involving the intravenous administration of radioactive melatonin to lactating rats (13). The observed rhythm of melatonin in the milk of rats also complements the initial report of Illnerova and co-workers (10), who described the appearance and rhythmicity of melatonin in human milk. It is interesting that the levels of melatonin in milk are similar to those in plasma. This contrasts with saliva melatonin, which is generally ~30% of the plasma concentration. Presumably both plasma protein-bound and free melatonin are transferred into milk in contrast to saliva (12). The dynamics of the endogenous milk melatonin rhythm together with its suppression by propranolol administration indicate that it could possibly act as an entraining signal for pups, thereby reflecting the circadian phase of the mother.

Is enough melatonin ingested to constitute a consistent signal? Not surprisingly, there are few reports on the oral bioavailability of melatonin in rat pups. In the present study, it was found that <5% of orally administered melatonin was circulating in the plasma of the rat pups within 30 min. In the only other study addressing this issue, Reppert and Klein (18) found that the amount of radioactive melatonin appearing in the plasma of rat pups 15 and 60 min after intragastric administration accounted for 0.6% of the administered dose. The differences between these two studies may in part be explained by a more rapid absorption of melatonin into the circulation when delivered orally in water compared with the cow's milk infant formula delivered via a stomach tube (18). The dose of melatonin administered in the current study (43 pmol) resulted in a transient three- to sixfold elevation in circulating melatonin in the rat pups. In light of the maximum melatonin concentration measured in milk (357 ± 66 fmol/ml), even this seemingly low dose must be considered pharmacological, because it represents a hypothetical instant ingestion by a single pup of >100 ml of milk. A litter of 8–12 rat pups shares an estimated 40–60 ml of milk over a 24-h period (7, 9) with a significant proportion of the feeding occurring during the rest period when the endogenous melatonin production of the mother is low. Even in the unlikely event of a pup drinking 1 ml of milk rapidly (i.e., 17–9% of its body wt between postnatal day 0 and postnatal day 5) at night, the 357 fmol ingested could be expected to increase the blood levels in the pup by a maximum of 3–6%, that is, from 300 to 309–318 pM. Because the affinity of the melatonin receptors for melatonin is on the order of 20–50 pM, it is difficult to see how such a small change in blood melatonin concentration could have profound physiological effects.

Circulating melatonin levels in the neonate may be elevated after ingestion of milk if the pup metabolizes melatonin slowly. Weinberg and Gasparini (32, 33) demonstrated an inability of neonatal rats to metabolize melatonin and further reported a retention of this hormone in the brain and liver compared with adult rats. We found that 5-day-old pups were capable of metabolizing melatonin to 6-sulphatoxymelatonin and clearing the majority of an injected pharmacological dose of melatonin from the circulation within 1 h of administration. Plasma melatonin levels were, however, significantly elevated 2 and 4 h later. By 10 days of age, the clearance appeared faster. It therefore seems unlikely that a lack of metabolism and clearance of melatonin in the young rat pup would significantly exaggerate the effect of melatonin transferred to the pups via the milk.

If the dam was indeed passing melatonin to the neonate, we would expect to detect a day-night difference in plasma melatonin in rat pups. In the present study, there was no significant rhythm in plasma melatonin levels in neonates over the first 6 postnatal days. This contradicts the findings of Velazquez and colleagues (27), who reported that serum melatonin was significantly decreased in 10-day-old rat pups suckling pinealectomized mothers in light and darkness. They concluded that the difference was due to milk transfer of melatonin. Tang and Pang (24), however, demonstrated a lack of diurnal rhythmic circulating melatonin in the neonatal rat over the first postnatal week, and Zitouni and co-workers (36) similarly failed to find a difference in plasma melatonin levels between 9-day-old pups born to sham-operated and...
pineal glands and from postnatal day 3 onward to use individual blood samples. Accordingly, we detected melatonin in pineal glands and blood from birth. Rhythmicity was apparent in pineal glands at postnatal day 5 and in blood on postnatal day 6, which is in agreement with the enzyme results cited above.

**Perspectives**

A high-amplitude rhythm of melatonin in the milk of lactating rats was observed with concentrations similar to circulating plasma levels. On the basis of pharmacokinetic studies, the strong correlation between pineal and plasma melatonin in 1-to-5-day-old pups, and the effects of adrenergic manipulation of maternal pineal function, we conclude that maternally derived melatonin that is transferred via the rat milk is unlikely to provide a high-amplitude signal of entrainment to the offspring. This conclusion does not contradict the observation that daily administration of exogenous melatonin can entrain the neonatal rodent (8, 25). Instead, it suggests that the SCN rhythmity is set during the prenatal but not the postnatal period of development.

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