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Abstract

Circadian melatonin secretion is the best-known output signal from the circadian pacemaker in the suprachiasmatic nucleus that indicates internal conditions of the body. We have established a system that enables long-term monitoring of melatonin secretion by implanting a transverse microdialysis probe in or near the pineal gland in a freely moving mouse. This *in vivo* method enabled continuous measurement of melatonin secretion over a period of >20 days in individual CBA mice, with simultaneous recording of the locomotor activity. Pineal melatonin secretion was completely matched to the circadian change of locomotor activity, and for the light-induced phase shift, the shift of melatonin secretion was clearer than the shift of locomotor rhythm. This analysis allowed us to detect rhythm with a high sensitivity: two peaks of daily secretion were observed, with the first small peak at the day–night transition time and the second large peak at midnight. The large nighttime peak was suppressed by tetrodotoxin, a Na^+ channel blocker, and enhanced by both phenylephrine and isoproterenol, α- and β-adrenergic agonists, whereas daytime melatonin levels were not affected by tetrodotoxin infusion. This finding suggests that, in CBA mice, melatonin release at night is activated by adrenergic signaling from the superior cervical ganglion, but the enhancement of melatonin during daytime is not mediated by neuronal signaling.

Keywords

circadian rhythm, locomotor activity, microdialysis

Abbreviations

SCN, suprachiasmatic nucleus; CSF, cerebrospinal fluid; TTX, tetrodotoxin; ZT, Zeitgeber time; L, light, D, dark.

In mammals, most physiological and behavioral events are subject to well controlled daily oscillations, and these rhythms are generated by the circadian clock in the suprachiasmatic nucleus (SCN) (1, 2). Recent progress in the genetic dissection of the clock genes has provided an outline of the molecular machinery of clock oscillation: the generation of rhythm begins at the transcription/translation feedback loops of clock genes (3–6). The dynamics of this core oscillatory loop is now monitored in transgenic animals carrying the *mPer1* promoter driven luciferase reporter (7–9).

From the *Drosophila* to mammals, an astonishing feature in circadian biology is that the rhythm of this gene transcription is integrated to cell, tissue, and organ, and finally to coordinated temporal organization at the system level. Because the molecular monitoring of gene transcription is now available, real-time rhythm–effector analysis is inevitable to clarify the linkage from the molecular oscillator to the effector. Of the circadian effectors, the behavioral sleep–awaking rhythm is the best known, and locomotor activity is used as a noninvasive output showing the clear-cut circadian expression in living mice. However, the behavioral rhythm is only one output of the SCN oscillator, and the real-time nature of the time information with regard to the body system has not been previously addressed.

In our search for a measure of the circadian output that indicates internal conditions of the body, we focused on melatonin (*N*-acetyl-5methoxytryptamine), a hormone primarily secreted by the pineal gland during darkness. The daily rhythm of melatonin synthesis in rodents such as rats or hamsters is known to be strictly regulated by the circadian clock in the SCN, because it is absent after SCN destruction (10) and then restored after SCN transplantation (11). Moreover, the route by which the SCN regulates the pineal function is well documented: circadian or photic SCN signals pass through a multisynaptic noradrenergic autonomic pathway, and regulate the activity of a pineal rate-limiting enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase, at transcriptional and posttranscriptional levels (12, 13). Secreted melatonin transmits temporal information to the brain through melatonin receptors in the SCN and other brain regions (14) to mediate a variety of physiological responses (15–17). Thus, this system is one of the best characterized output systems of the circadian pacemaker that indicates internal conditions of the body.

To reveal the circadian mechanism from genes to output system, we tried to develop a continuous recording of melatonin in mice, a species for which the genetic

nature of circadian rhythm is well understood. However, monitoring melatonin in mice can be difficult because most strains of inbred mice have lost the ability or are very poor at synthesizing melatonin (e.g., C57BL/6, BALB/c, and ARK). However, two strains, CBA and C3H, that possess the enzymes for melatonin synthesis display a stable and reproducible circadian rhythm (18–21). However, existing data on mouse melatonin have been obtained from homogenated pineal tissues or peripheral blood, thus making it very difficult to continuously monitor the hormone.

To establish a method of continuous monitoring, therefore, we applied the brain microdialysis technique to assess a 24-hr pattern of endogenous melatonin secretion in individual awake mice. In the pineal gland of rats, the microdialysis technique has been applied in attempts to monitor a 24-hr pattern of melatonin (22–25). However, implanting the microdialysis probe directly into the very tiny pineal gland of a mouse is more difficult. Interestingly, Skinner and Malpaux (26) have recently found in sheep that melatonin enters the ventricular system directly from the pineal gland and melatonin concentrations in the ventricular cerebrospinal fluid (CSF) are several times higher than blood melatonin levels. Encouraged by this report, we thought that the above difficulty might be alleviated through the implantation of the probe into the CSF of the ventricular system near the pineal body, which would be much easier than probe implantation into the pineal itself while still enabling us to detect melatonin in the CSF. Through the long-term implantation of the dialysis probe in or adjacent to the pineal gland, optionally in combination with locomotor measurements, in the present study, we have discovered a bimodal daily pattern of melatonin secretion caused by synaptic and nonsynaptic mechanisms.

Methods

Subjects.

Male CBA mice (Japan SLC, Hamamatsu, Japan), one of the most melatonin-proficient strains among a variety of inbred mouse strains, weighing 24–32 g at the time of surgery, were used. The animals were individually housed under a 12-hr light (L)/12-hr dark (D) lighting schedule (lights on at 0700 hours, lights off at 1900 hours) in a temperature-controlled environment (23–25°C) for at least 2 weeks before the experiment; food and water were available ad libitum. All of the procedures for animal

treatment and surgery were in accordance with the guidelines established by the Institute for Experimental Animals of Hamamatsu University School of Medicine and were approved by the University Committee for Animal Experiments.

Surgery and Microdialysis.
The experiments were performed with transverse probes, which were constructed as described by Drijfhout *et al*. (24) with a minor modification. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a Narishige apparatus with skull level between bregma and lambda. One hole was made on each side of the skull (0.0 mm anterior and 0.3 mm ventral to lambda) with a dental drill and a dialysis tube (0.24-mm o.d.; 0.01-mm wall width; 5,000 MW cut-off; Cuprophan hollow fiber, Eicom, Kyoto, Japan) was transversely inserted in the pineal gland under the guidance of a stainless steel wire attached in a horizontal position to a stereotaxic holder. The surface of the dialysis membrane was glued with an epoxy resin (Araldite Rapid; CIBA-GEIGY) except for a 1-mm-wide center zone. The mice were allowed at least 1 week to recover from the surgery. Thereafter, the animals were moved to a 30 × 30 × 35-cm transparent acrylic test box, the floor of which was covered with sawdust-like paper. The transverse probe was connected through a dual-channel liquid swivel (Instech, model 375/D/22QM, Plymouth Meeting, PA) with PE-10 polyethylene tubing to a microinfusion pump (Univentor, Zejtun, Malta) and a sample collector equipped with a refrigerator (Eicom, EFC-82). The probe was perfused with a Ringer's solution (138 mM NaCl/2.4 mM KCl/1.2 mM $CaCl_2$, pH 7.0) at 1 μl/min. After a period of several days of habituation to the box and perfusion line, daily samples were automatically collected every hour in a small vial and were analyzed off-line by HPLC-fluorescence detection. The *in vitro* recovery of melatonin with our probes was 15.3±0.9% (mean±SEM, n=6) at 37°C. In everyday life, the mice had free access to food placed on the floor of the test box and to water given from the lid of the box. Daytime illumination measured at the level of the floor was 120–150 lux. During nighttime, there was a continuous dim red light of <1 lux.

Locomotor Activity. Simultaneously with melatonin monitoring, the locomotor activity in individual mice was also detected with an infrared sensor (Biotex, BS-002, Kyoto,

Japan) placed 35 cm above the floor of the box, and recorded each hour by a personal computer.

Drugs. Tetrodotoxin, cycloheximide, phenylephrine, isoproterenol, prazosin, and propranolol were purchased from Sigma. Drugs were dissolved directly into the Ringer's solution.

Statistical Analysis. The data were expressed as mean±SEM values and statistically evaluated by using the Student's paired *t* test, unless stated otherwise. *P* values <0.05 were regarded as significant.

Histology. At the end of the experiment, the mice were decapitated and the brain was dissected with the pineal gland fixed in 10% formaldehyde. After decalcification and paraffin embedding, saggital sections (6 μm) of the pineal were cut and stained with Cresyl Violet, Klüver–Barrèra, and Holzer to determine the location of the probe and the change in the tissue surrounding it.

Results

The Transverse Microdialysis Probe Could Detect Stable Rhythmic Melatonin Secretion Over 100 Days in an Individual CBA Mouse.

By applying the transverse microdialysis probe to the brain of the CBA mice, we could successfully detect a clear melatonin rhythm, as we expected based on the day–night difference of melatonin content in homogenates. To analyze the circadian rhythm through this method with the melatonin secretion as a marker, we had to determine the consistency and strength of the daily melatonin secretion cycle over a long period. Fig. 1 shows an example of repeated microdialysis in the pineal gland of a mouse. We clearly detected a stable rhythmic secretion of melatonin for up to 169 days, and this demonstrates that our method is effective for analyzing the circadian rhythm using this marker of the body. The peak amplitudes of melatonin in a light–dark cycle were not different from those in a constant darkness (data not shown).

The above data are not exceptional; in the present study, we permanently implanted the transverse probe in a total of 40 mice. Fourteen of the implanted animals

were unusable, mainly because of probe misplacement, and the remaining 26 animals could be used in the experiments. In 20 of these animals, from which the present data were obtained, the probes stayed functional for at least 20 consecutive days. The average available period of these probes until termination of the experiments was 66±11 days (n=20).

Why did such clear melatonin rhythms continue in the transverse-microdialysis probe? We performed a histological examination of 10 mice, and found that, in five of them, the probes were within the pineal gland (we thus refer to these as transpineal probes). In these cases, there was a thin layer of polymorpho- and mononuclear leukocytes infiltration (Fig. 2A), but not of fibrous or glial scar tissue (Fig. 2C and E), around the tubing. Also, very little reaction (Fig. 2B, D, and F) was found in the five other mice where the probes were in the CSF adjacent to the pineal (thus referred to as transventricular probes). As should be expected, the mean peak value of the melatonin concentrations was much higher in the transpineal probes (n=5, 1.78±0.37 ng/ml dialysate) than in the transventricular probes (n=5, 0.48±0.13 ng/ml dialysate) (t=3.35, P<0.05, df=8). For either type of probe, note that the daily peak-trough levels of melatonin did not diminish after a prolonged period (Fig. 2G and H), thus indicating that this technique is suitable for analyzing the circadian profiles of the animals.

Circadian Secretion of Pineal Melatonin Correlates with the Rhythm of Locomotor Activity in Individual CBA Mice.

Because the above technique enabled us to obtain the circadian profiles of melatonin secretion, we next compared this secretory pattern with circadian profiles of locomotor activity, another index of circadian rhythms. To do this, we put the dialyzed CBA mice into a locomotor cage, in the dialysates, and the locomotor activity in the same mouse over 10 consecutive days under a 12-hr L/12-hr D cycle. Fig. 3 shows the mean circadian pattern of each parameter over 10 consecutive days for the six mice. When mice were entrained to this LD cycle, melatonin levels and activity counts both showed a robust and reproducible rhythm with distinct peak amplitudes. In all of the animals, melatonin levels rose steadily throughout the light period and reached a small circadian peak in the hour after lights-off. A second, larger increase appeared late in the dark period. Thus, melatonin levels exhibited a bimodal pattern with the first peak appearing at the start of darkness and the second large peak occurring near the end of

the night. The grand average of the amplitude of the first small and second large peaks in the melatonin concentrations were 0.21±0.01 and 0.75±0.01 ng/ml dialysate, respectively, over 10 experimental days. Activity counts also demonstrated a similar rhythmicity, but with a large peak appearing immediately after dark.

Circadian Melatonin Secretion Is Clock-Controlled and Synchronized with Locomotor Activity.

To test whether the melatonin peak is driven by an endogenous clock, we examined the secretory profile of melatonin in free-running conditions or after brief exposure to light. Fig. 4 illustrates the individual pattern of melatonin and simultaneously recorded locomotor rhythms over a total of 25–30 consecutive days for three animals. When the lighting schedule was switched from 12 hr L/12 hr D to a DD cycle, the dialysate levels of melatonin and locomotor activity free-ran correspondingly. On the other hand, the two peaks that appeared early and late at night under a normal LD condition persisted even in constant darkness. Melatonin secretion and locomotor activity under the DD condition both exhibited a stable free-running rhythm, with their peak amplitudes being similar to those under the LD condition. We examined the periods of the two parameters for each period of 7–10 consecutive days for six mice under the entrained and free-running conditions. Entrained rhythms under 12 hr L/12 hr D conditions had a period near 24.0 hr for both melatonin levels and locomotor activity in all six of the animals. The free-running rhythms for both the melatonin concentrations and locomotor activity had also a nearly identical period (≈23.7 hr). The periods were significantly shorter in free-running compared with entrained situations (melatonin: t=4.78, df=5, P<0.01; locomotion: t=3.5, df=5, P<0.05).

Next, we investigated how a brief light pulse affected melatonin levels in dialysates sampled from transverse probes in the CBA mice. Light pulses are known to instantly suppress the nocturnal melatonin secretion in the rodent pineal tissue (20, 27). When the animals in our experiment were exposed to a 15-min light pulse at circadian time (CT) 20, dialysate melatonin levels significantly decreased to basal levels within 2 hr after the start of light exposure (t=5.13, df=4, P<0.01) **(**Fig. 5A). In contrast, locomotor activity levels abruptly and significantly increased in response to light exposure (t=3.15, df=4, P<0.05) (Fig. 5B). Light exposure during subjective night is

also known to induce a phase-shift of circadian locomotor activity. Indeed, in these animals, on the application of the brief light at CT 20, an advance phase, the locomotor activity showed a distinct phase advance after 2 days (Fig. 5B). The phase-advance of the melatonin rhythm was more immediate, and a phase-advance by ≈1 hr was clearly noted, in the large night peak (sign test: df=4, P=0.032) but not the small peak (df=4, not significant), even by the day after the light exposure (Fig. 5A).

Tetrodotoxin Suppresses the Night Increase but Not the Day Increase of the Circadian Melatonin Secretion.

In this study, we have described the bimodal pattern of melatonin secretion into the CSF: a small day–night transition peak and a large midnight peak. Evidence suggests that melatonin secretion in other species, including rats and hamsters, is regulated by innervation from the superior cervical ganglion (28). Thus, to reveal any neuronal regulation of the melatonin secretion in CBA mice, we examined whether the two melatonin increases were affected by the application of tetrodotoxin (TTX), a Na^+ channel blocker.

Infusion with TTX (10 μM) through the transverse probe for 3 hr from Zeitgeber time (ZT) 18 to ZT 21, when the large night peak began under normal untreated conditions, immediately and significantly decreased melatonin concentrations (Fig. 6B; t=3.55, df=4, P<0.05). This result is consistent with the effect of TTX on rat melatonin secretion, a species for which only one night peak is observed (24), and suggests that the large midnight melatonin peak is evoked by the influence of neuronal inputs.

We next infused TTX through the transverse probe for 3 hr from ZT 6 to ZT 9, at which time melatonin steadily increased under the control conditions. However, this treatment caused no significant change in melatonin concentrations (Fig. 6A; t=1.18, df=5, not significant). This observation suggested that the constitutive elevation of melatonin secretion throughout the light period is not caused by synaptic inputs.

Is the steady increase of pineal melatonin secretion thus accompanied by increased synthesis of the proteins, or does it only reflect a gradual increase in the secretion of melatonin? We applied cycloheximide, an inhibitor of protein synthesis, during the daytime, and measured melatonin levels in the dialysates. During the light phase, infusion with 35 μM cycloheximide for 11 hr from ZT 1 to ZT12 resulted in a

significant decrease in melatonin concentrations (Fig. 6C; t=3.88, df=5, $P < 0.01$). This finding suggests that the concomitant increase of melatonin secretion during daytime is not a simple diffusion increase, but accompanies the protein production that involves melatonin biosynthesis. The night-peak suppression by cycloheximide observed at ZT 19–23 might be derived from a blocking of the production of enzymes involving melatonin synthesis, which has been similarly observed in rats (29).

Adrenergic Signaling Regulates Melatonin Secretion at Midnight.

Because the late night melatonin peak is regulated by neuronal input, we characterized the chemical nature of this input. Because melatonin secretion in the rodent pineal is modulated by the noradrenergic system (28), we determined whether noradrenaline stimulates nocturnal melatonin secretion through a dual α- and β-adrenergic mechanism. By infusion of 100 μM phenylephrine, the α-adrenergic agonist, from ZT 18 to ZT 21, melatonin levels began to increase immediately after the application, rose consistently, and then reached a maximal value at the end of the application (3 hr after the start of infusion) (Fig. 6D; t=2.57, df=5, P<0.05). Cessation of phenylephrine treatment rapidly decreased melatonin concentrations, and the levels returned to vehicle-treated control levels after 1–2 hr. On the other hand, by infusion of 100 μM isoproterenol, the β-adrenergic agonist, melatonin concentrations were not altered for 2 hr in the beginning of the application, but began to increase thereafter. The increased levels of melatonin persisted even after cessation of the application and reached a peak value 2 hr later (5 hr after the start of infusion) (Fig. 6E; t=3.44, df=5, P<0.001). Even 3 hr after the end of isoproterenol treatment, higher levels of melatonin were observed in comparison to vehicle-treated control levels. In addition, increased levels of melatonin at night were largely suppressed when either 100 μM prazosin, the α-adrenergic antagonist, or 100 μM propranolol, the β-adrenergic antagonist, were added to the perfusion medium for 4 hr from ZT 10 to ZT 14 (n=3, data not shown). These findings indicate that the melatonin concentration of a CBA mouse is differentially modulated by noradrenaline through the α- and β-receptor.

Discussion

Transverse, Either Transpineal or Transventricular, Probes Allow Long-Term Monitoring of Mouse Pineal Melatonin.

This microdialysis study has examined the bimodal secretion of melatonin from the pineal gland during light and dark phases of a 24-hr period in individual CBA mice. Transverse probes, which were permanently implanted into the pineal gland *per se* (transpineal probes) or in the ventricular CSF adjacent to it (transventricular probes), enabled us to continuously record the 24-hr rhythm of melatonin in the same animal over at least 20 days and repeatedly up to 169 days. Histological examination after the experiments revealed little tissue reaction on the probe membrane. When probes are chronically implanted in the brain, tissue reactions that gradually occur on the dialysis membrane are thought to form a diffusion barrier, thus leading a decreasing amount of the target substance in dialysates over time (30). To our surprise, however, our probes remained functional for an average of 66 days. Why was such a long-term dialysis possible with transpineal or transventricular probes? A major reason might be that a transventricular probe embedded in the CSF causes little damage to the pinealocytes and no tissue reaction on the membrane. In addition, even if a diffusion barrier were formed on the membrane, melatonin is highly lipophilic so it can easily enter the membrane through such a barrier. Consistent with the evidence of our results, in a recent microdialysis study in rats approximately half of the transpineal probes stayed functional for 2 weeks or longer (31).

Light Induces Immediate Phase Shifts of Circadian Melatonin Rhythms.

This study reports on the relationship between the melatonin secretion rhythm and the locomotor activity rhythm. Through our method, melatonin levels were monitored by using the transverse probe, and this permitted long-term collection of hourly dialysate samples from a single animal. Together with melatonin secretion, we could simultaneously monitor circadian locomotor activity. The circadian rhythms of pineal melatonin concentrations and locomotor activity were closely coupled under both normal LD and continuous dark conditions over long periods of time, supporting the hypothesis that the two overt rhythms are regulated by a common or very similar timing mechanism under physiological conditions (32).

In this study, we found that melatonin levels displayed a clear phase-advance from the next cycle, although the rhythm of the locomotor activity takes a few more

days to exhibit an explicit phase advance. When a phase shift of the clock will be complete after exposure to a light pulse is an important question, and a two-light-pulse paradigm study of locomotor activity strongly suggests that the phase-resetting of the mammalian pacemaker is complete within several hours (33). This has also been supported by a recent finding at the clock gene level showing that the application of *N*-methyl-D-aspartate, an *in vitro* counterpart of a light-pulse in behavior, into the SCN slice of transgenic mice carrying the *mPer1* promoter driven luciferase gene, induced the phase-shift of the *mPer1* transcription rhythm within 2–3 hr (7). These studies strongly suggest that the clock will instantly change the phase after a light pulse. Thus, melatonin secretion in the pineal gland should be a more reliable phase marker of pacemaker activity in the SCN than the locomotor activity rhythm.

Daytime and Nighttime Melatonin Secretion Is Differentially Regulated.

Through our success in the continuous recording of melatonin secretion from the pineal gland, we made several findings. First, during the daytime, melatonin secretion started to increase at dawn, continued to steadily increase, and reached a peak right after dark. Second, at midnight, the melatonin concentration sharply increased and reached a peak 9–11 hr after the beginning of dusk. The melatonin concentration rapidly decreased afterward, reaching its lowest value at close to dawn. Such a circadian pattern in CBA mice was consistent with the tissue concentrations of melatonin in B6D2F_1 mice (34).

Because melatonin concentrations in CBA mice have two daily peaks (the small peak during day–night transition and the large peak at night), we examined whether these increases were neuronal in origin. TTX infused through the probe by reverse dialysis greatly reduced melatonin concentrations at night, but not during the day. The failure of melatonin levels to fall when TTX blocked the impulse flow suggests that the daytime rise in the melatonin concentration was mostly caused by endogenous activation of melatonin biosynthesis inside the pinealocyte (34, 35). This suggestion is strongly supported by evidence indicating decreased melatonin secretion by the translation inhibitor, cycloheximide.

On the other hand, the reduction in melatonin levels during TTX infusion was consistent with a previous microdialysis result in rats which indicated that the rise in the night melatonin concentration depends on the conduction of impulses from sympathetic

nerves originating in the superior cervical ganglion (24). The pinealocytes are modulated by noradrenergic neurons via two subtypes of adrenergic receptors, α- and β-receptors (12, 24, 36, 37). Stimulation of the pineal gland by the α-adrenergic agonist phenylepherine produced a rapid and transient increase in melatonin levels. On the other hand, a delayed and prolonged increase in melatonin was observed after infusion of the adrenergic β-agonist isoproterenol. Moreover, the nighttime increase in melatonin concentrations was blocked by both the α-adrenergic antagonist prazosin and the β-receptor antagonist propranolol. These results indicate that, as observed in rats, a midnight accumulation of melatonin in a CBA mouse occurs under the modulation of noradrenaline through the α-receptor and the β-receptor. In addition, the time-course difference in the effects of phenylephrine and isoproterenol may indicate that in the mouse, the former drug influences arylalkylamine *N*-acetyltransferase activity primarily at a posttranslational level, whereas the latter acts through transcriptional mechanisms.

In conclusion, we used a transverse probe implanted into the pineal body or into its adjacent CSF of the cisterna superior to establish continuous and chronic monitoring of melatonin levels in the dialysates of individual living CBA mice. We also demonstrated that melatonin of nonexocytotic origin in the mouse pineal gland is circadianly secreted during daytime, as well as at nighttime, when the secretion depends on adrenergic signaling from the superior cervical ganglion. Thus, this approach should provide a useful means of evaluating the short-term and long-term *in vivo* effects of various drugs or photoperiod alterations on the SCN pacemaker in mice through the measurement of melatonin concentrations. Furthermore, microdialysis using mice in which the putative clock or clock-controlled genes are mutated, but that have melatonin in their pineal gland, would also provide a valuable tool for determining how these genes regulate melatonin secretion.

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Figure legends

Fig. 1. Atypical example of repeated microdialysis over a 169-day period in the same mouse with a chronically implanted transverse probe. The mouse was dialyzed continuously from day 1 to day 32 (for 32 days), from day 47 to day 54 (for 8 days), from day 77 to day 79 (for 3 days), then from day 91 to day 169 (for 79 days). Rest periods of 11–22 days separated the four continuous dialysis sessions. Lighting conditions were 12-hr L/12-hr D except for constant darkness from day 11 to day 32 (22 days), from day 49 to day 54 (6 days), and from day 135 to day 168 (34 days). There was no consistent relationship between a normal LD cycle and a constant darkness with respect to the amplitude of nocturnal melatonin secretion. The dark periods are indicated in gray.

Fig. 2. (A) Sagittal brain section with dialysis tubing (arrows) positioned in the pineal gland for 27 days. There is small inflammatory reaction around the tubing. Cresyl Violet staining was used. (B) Sagittal section with dialysis tubing positioned in the CSF of the ventricular system adjacent to the pineal gland for 47 days. The ventricular system shows little reaction. Cresyl Violet staining was used. (C and D) No fibrious reaction around either the transpineal or the transventricular probe. Klüver–Barrèra staining was used. (E and F) No glial reaction around either the transpineal or the transventricular probe. Holzer staining was used. (G and H) Comparison of melatonin levels in dialysates from transpineal and transventricular probes. cx, cerebral cortex; ic, inferior colliculus; pi, pineal gland; s, skull; sc, superior colliculus. (Scale bar=500 μm.)

Fig. 3. Daily profiles of melatonin levels and locomotor activity for six individual mice maintained in the 12-hr L/12-hr D cycle. Data are expressed as the mean±SEM of 10 consecutive days for each mouse. The dominant circadian period of melatonin and locomotor rhythms of each mouse was ≈24 hr (P<0.05) according to χ^2-periodgram analysis. A 12-hr rhythmic component was also statistically validated (P<0.05). The dark periods are indicated in gray.

Fig. 4. A double plot of melatonin levels and locomotor activity for three mice exposed to both 12-hr L/12-hr D and DD cycles. To characterize the free-running rhythms of

melatonin secretion and locomotion, mice were first entrained to a cycle of 12-hr L/12-hr D for 7–10 days, and then exposed to constant darkness (DD) for 15 days or longer. The time of day is indicated horizontally, and consecutive days are indicated vertically. The horizontal open bar at the top of each panel shows the light period, and the filled bar shows the dark period. Data are graphed as color diagrams.

Fig. 5. Daily rhythms of melatonin levels before and after five mice were exposed to light. To investigate the effects of a brief light pulse on melatonin (A) and activity (B) levels, we exposed the mice to fluorescent light (300 lux) for 15 min at circadian time 20. In this situation, the lights remained off for the remainder of the experiment. Data are mean±SEM (bars) values. The broken line represents a control based on data from the day before light exposure. Arrows indicate the time of light-pulse exposure. (Inset) Graphs show the area under the curve (AUC) levels for melatonin (A) and locomotion (B) at ZT 20–21. *, $P<0.05$; **, $P<0.01$; significantly different from controls.

Fig. 6. (A and B) Effects of TTX infusions during the day (A; $n=6$) or at night (B; $n=5$) on dialysate melatonin concentrations. To examine whether daily changes in dialysate melatonin depended on neuronal activity, we infused 10 μM TTX through the probe from ZT 6 to ZT 9 or from ZT 18 to ZT 21 during the 12-hr L/12-hr D cycle. (Insets) Graphs show the area under the curve (AUC) levels for daytime melatonin (A) at ZT6–9 and for nighttime melatonin (B) at ZT18–21. (C) Effect of cycloheximide (Cyc) infusions during the day ($n=6$) on dialysate melatonin concentrations. To examine whether daytime increases in dialysate melatonin depended on protein synthesis within the pinealocyte, 35 μM cycloheximide was infused through the probe from ZT 1 to ZT 12 during the 12-hr L_12-hr D cycle. (Inset) Graph shows the AUC levels for melatonin at ZT 1–12. (D and E) Effects of phenylephrine (Phe) (D; $n=6$) or isoproterenol (Iso) (E; $n=5$) infusion on dialysate melatonin concentrations. To explore the effects of noradrenergic drugs on melatonin concentrations, we infused 100 μM phenylephrine or 100 μM isoproterenol through the probe from ZT 18 to ZT 21 during the 12-hr L/12-hr D cycle. (Inset) Graph shows the AUC levels for melatonin at ZT 18–21. In these experiments, the same mice were used at different days to obtain vehicle and drug-treatment values. Data are mean±SEM (bars) values. The solid bar indicates the

period of infusion. The dark periods are indicated in gray. *, $P<0.05$; **, $P<0.01$; significantly different from vehicle (Veh) controls.

Fig. 1

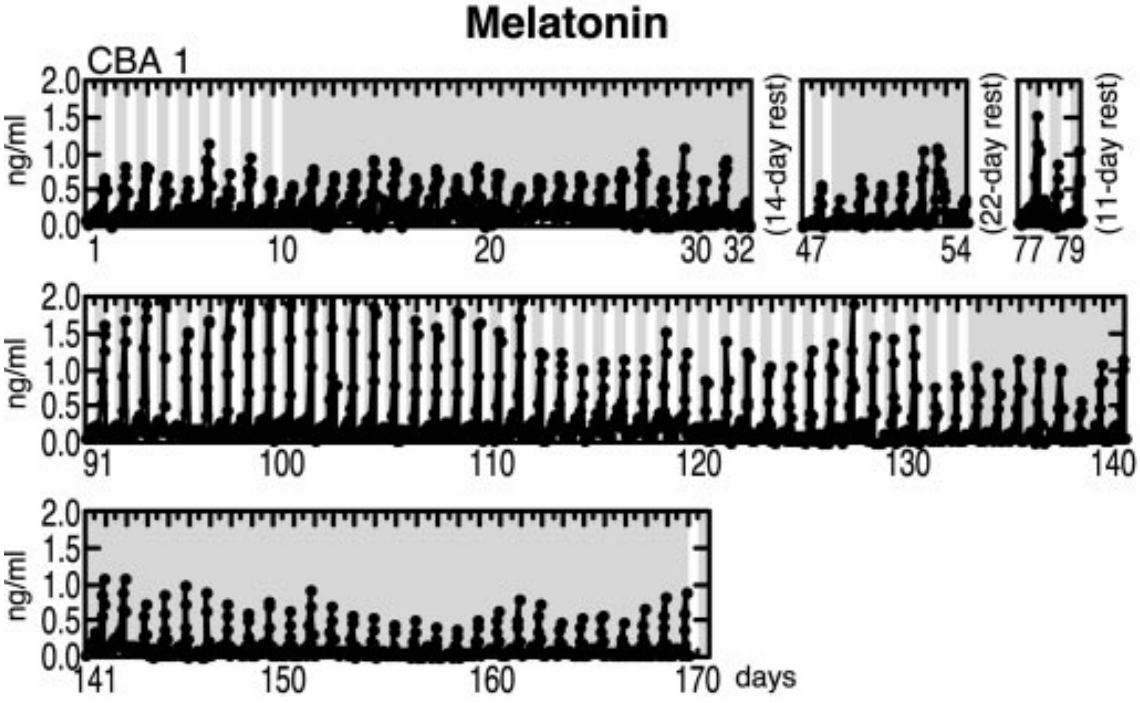

Melatonin
CBA 1
ng/ml
2.0
1.5
1.0
0.5
0.0
1
10
20
30 32
(14-day rest)
47
54
(22-day rest)
77 79
(11-day rest)
91
100
110
120
130
140
141
150
160
170
days

Fig. 2

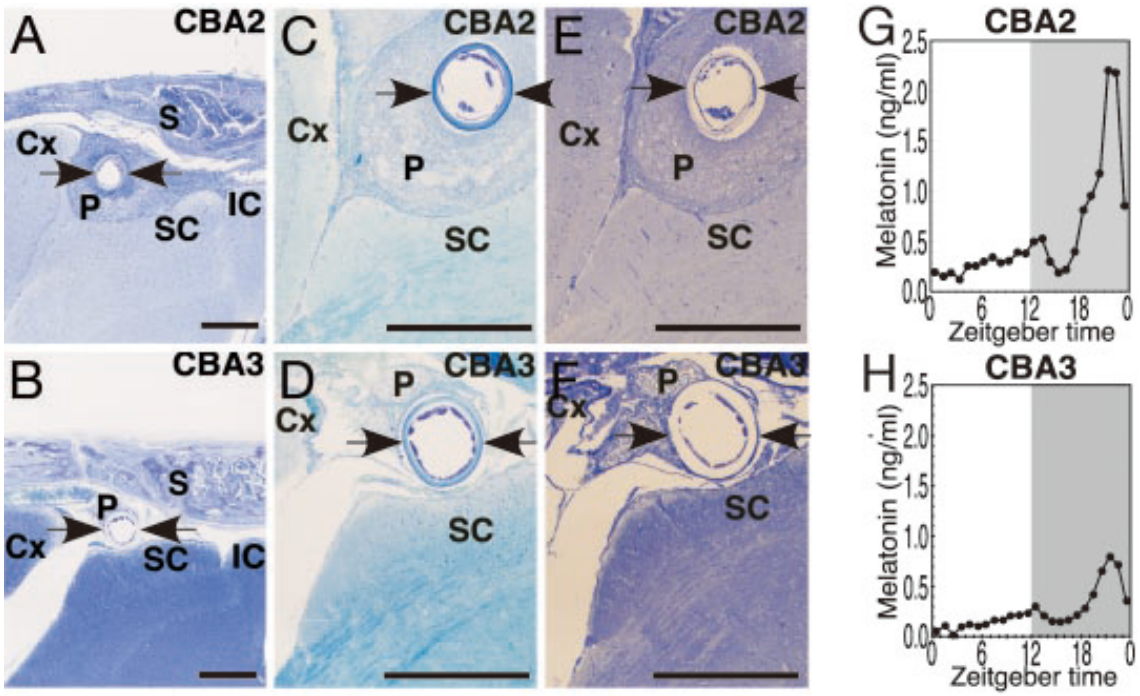

A
CBA2
S
Cx
P
SC
IC
C
CBA2
Cx
P
SC
E
CBA2
Cx
P
SC
B
CBA3
S
P
Cx
SC
IC
D
CBA3
P
Cx
SC
F
CBA3
P
Cx
SC
G
CBA2
Melatonin (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
18
0
Zeitgeber time
H
CBA3
Melatonin (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
18
0
Zeitgeber time

Fig. 3

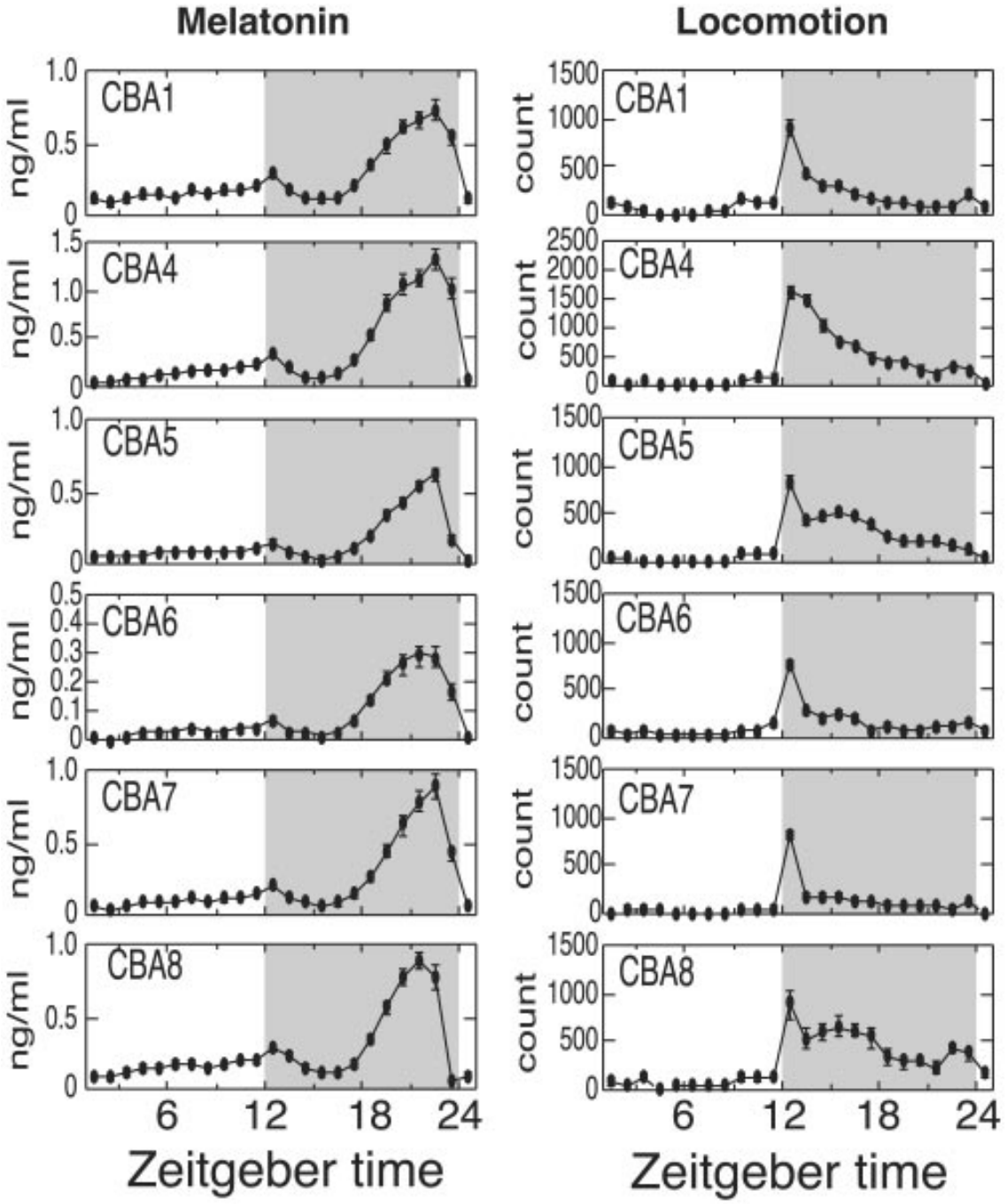

Melatonin
Locomotion
CBA1
CBA4
CBA5
CBA6
CBA7
CBA8
ng/ml
count
Zeitgeber time
6
12
18
24

Fig. 4

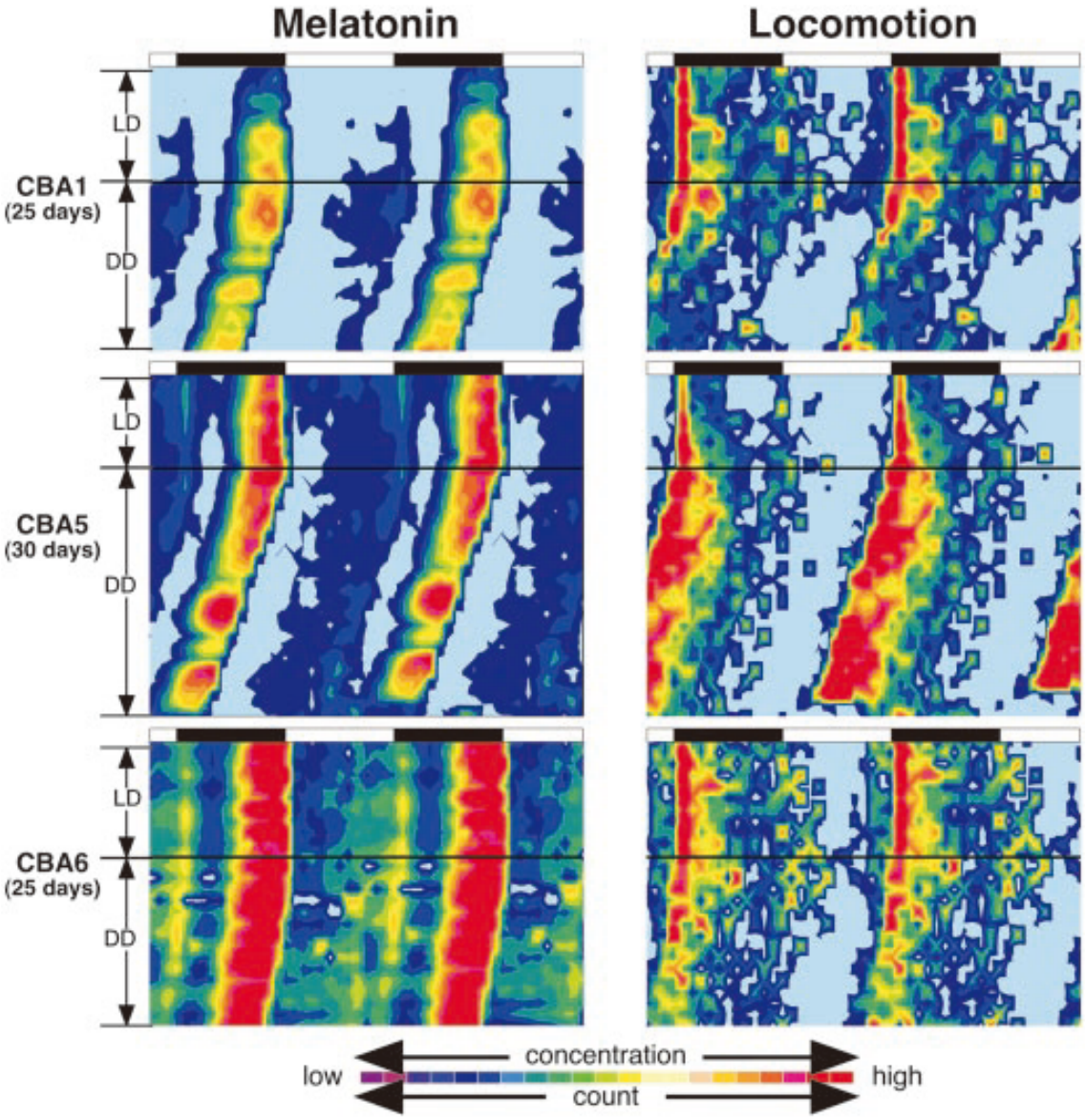

Melatonin
Locomotion
LD
CBA1
(25 days)
DD
LD
CBA5
(30 days)
DD
LD
CBA6
(25 days)
DD
concentration
low
high
count

Fig. 5

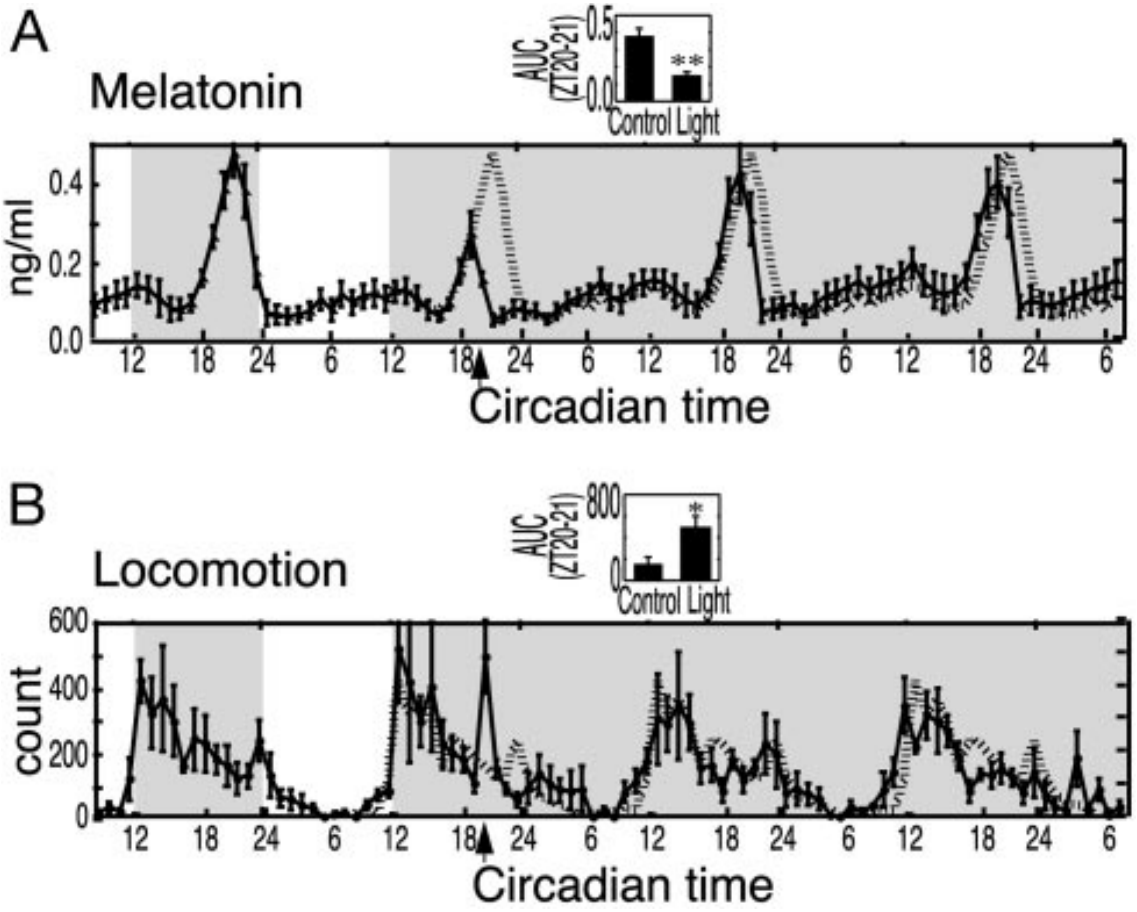
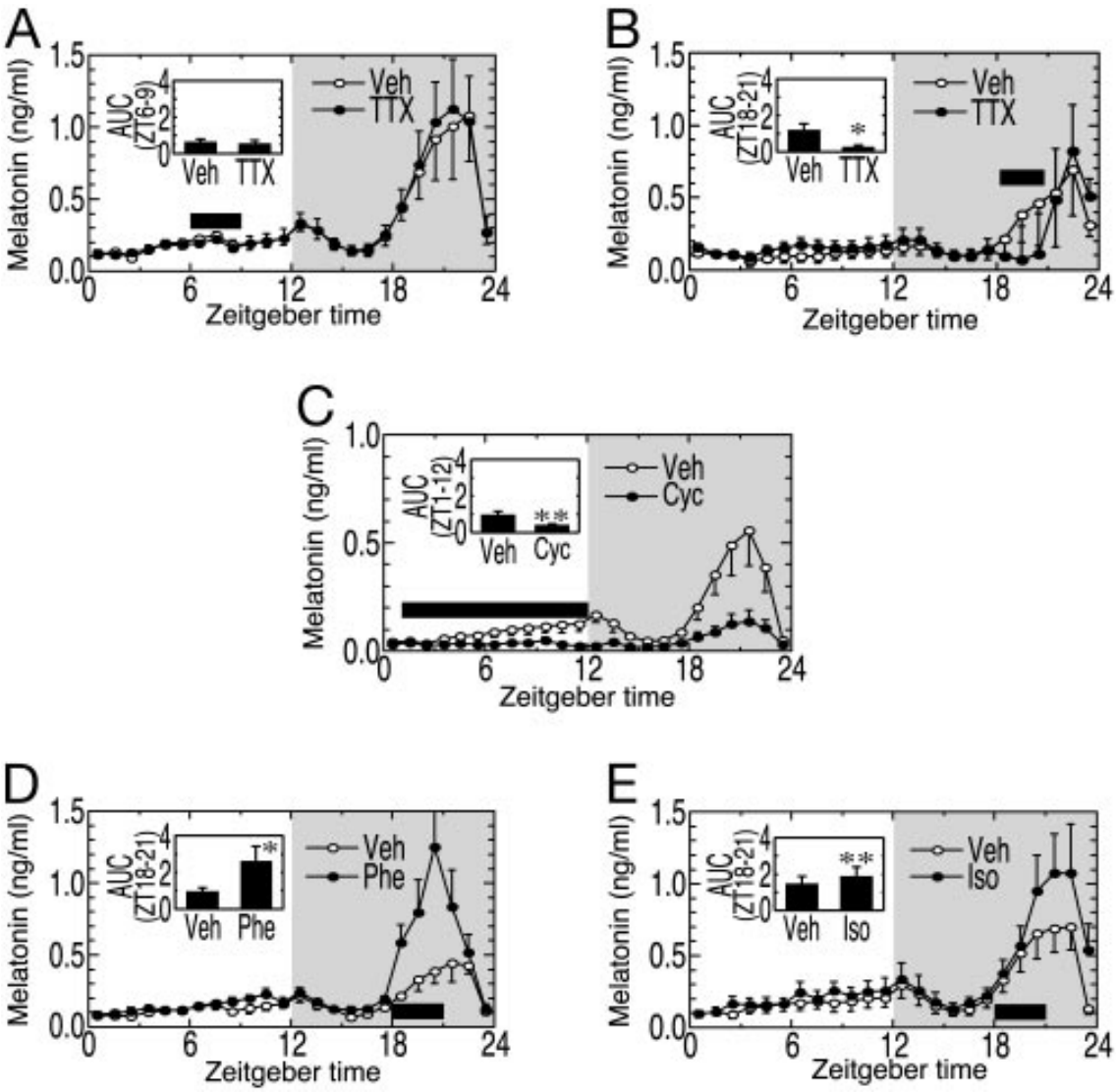

A
Melatonin
AUC (ZT20-21)
0.5
0.0
**
Control Light
ng/ml
0.4
0.2
0.0
12 18 24 6 12 18 24 6 12 18 24 6 12 18 24 6
Circadian time
B
Locomotion
AUC (ZT20-21)
800
0
*
Control Light
count
600
400
200
0
12 18 24 6 12 18 24 6 12 18 24 6 12 18 24 6
Circadian time

Fig. 6


A
Melatonin (ng/ml)
1.5
1.0
0.5
0.0
0
6
12
18
24
Zeitgeber time
Veh
TTX
AUC (ZT6-9)
4
2
0
Veh
TTX
B
Melatonin (ng/ml)
Zeitgeber time
Veh
TTX
AUC (ZT18-21)
*
Veh
TTX
C
Melatonin (ng/ml)
1.0
0.5
0.0
Zeitgeber time
Veh
Cyc
AUC (ZT1-12)
**
Veh
Cyc
D
Melatonin (ng/ml)
Zeitgeber time
Veh
Phe
AUC (ZT18-21)
*
Veh
Phe
E
Melatonin (ng/ml)
Zeitgeber time
Veh
Iso
AUC (ZT18-21)
**
Veh
Iso