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Interactive report

Melatonin promotes sleep-like state in zebrafish[1]

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Abstract

The sleep-promoting effect of the pineal hormone melatonin in humans is known for decades. However, the mechanisms of this phenomenon remain obscure, mainly due to lack of a simple, genetically tractable, animal model. We now report that melatonin promotes sleep-like state in a diurnal lower vertebrate, zebrafish (*Danio rerio*), and this effect is mediated through activation of specific melatonin membrane receptors. Furthermore, our data show that the sleep-like state in zebrafish has fundamental similarities with sleep in mammals, including characteristic postures, elevated arousal threshold to sensory stimulation and a compensatory rest rebound following rest deprivation, and can be induced by conventional hypnotics, diazepam and sodium pentobarbital. Collectively, these data indicate that melatonin is evolutionary conserved sleep-promoting agent in diurnal species and suggest that zebrafish provide an efficient animal model for studying the molecular mechanisms of sleep regulation and for screening new types of hypnotic medications.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Melatonin; Sleep; Zebrafish; Receptor; Benzodiazepine; Barbiturate

1. Introduction

The daily rhythm in circulating melatonin, which is low during the day and high at night, might be a significant physiological factor in sleep initiation and maintenance in humans. The period of increased melatonin secretion from the pineal gland is concurrent with the habitual hours of sleep in humans [10], and the onset of melatonin secretion highly correlates with the onset of the evening sleepiness [1,17,21]. Furthermore, administration of melatonin in doses that result in physiological circulating levels of the hormone promote sleep in humans [5,14,21] and diurnal non-human primates [20]. Conversely, in nocturnal species, e.g., rats or mice, nighttime melatonin secretion coincides with the activity phase of their 24-h rest/activity cycle and, in these animals, administration of physiological or low pharmacological melatonin doses does not promote sleep [11,16].

The mechanisms responsible for the sleep-promoting effects of melatonin in humans remain unknown. In search of a simple vertebrate animal model for studying the effects of melatonin on sleep, we chose to investigate the zebrafish, a diurnal vertebrate with clear daytime activity and nighttime rest and robust circadian pattern of melatonin secretion [2,9]. Given the accumulated experience in conducting large-scale genetic screens in zebrafish, the multiple mutant phenotypes available and the current construction of genetic and physical maps of this lower vertebrate, zebrafish is one of the best candidates for studying the mechanisms of homeostasis and the molecular bases of behavior in diurnal vertebrates [7].

Sleep state is defined using both behavioral and electrophysiological criteria [4]. The patterns of brain electrical activity characteristic of human sleep have been documented only in other mammals and in birds. In contrast, it

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was suggested that typical behavioral features of sleep might be common in the animal kingdom, including prolonged quietness associated with specific postures, elevated arousal threshold, increase in the amount of sleep and heightened arousal threshold after sleep deprivation (sleep rebound) or after treatment with sleep-promoting agents [3,8]. Prior to initiating studies on possible effects of melatonin on sleep-like state in zebrafish, we first had to determine the extent to which the behavioral manifestations of the rest state in this lower vertebrate are comparable with well-studied mammalian behavioral sleep characteristics.

The present study examined the similarities between rest state in zebrafish and sleep in mammals, and the effects of melatonin on sleep-like state in this diurnal lower vertebrate. In addition, the mechanisms of melatonin sleep-promoting effect were studied using specific melatonin and benzodiazepine receptor antagonists.

2. Material and methods

2.1. Animals

Larval zebrafish (*D. rerio*, Tubingen strain), 7–14 days old, were maintained at 23°C, under 12:12 light:dark cycle, with zeitgeber time (ZT) 0 corresponding to lights on time, and were fed *Paramecium* daily.

2.2. Locomotor activity

The locomotor activity was registered in constant darkness, using an automatic image analysis system, similar to one described earlier [2]. The images of the fish moving in the individual square 650 μl wells of a microplate (Poly-Filtronics, Rockland, MA), backlighted by an 880 nm infra-red (IR) source, were transmitted from an IR camera (Sanyo VDC-9212, ADI, Woburn, MA) via a TV tuner (AverTV USB, AverMedia) to a Pentium III 667 computer. Our custom software program, FishWatch, continuously monitored 60 fish and stored in separate files the distance traversed by each fish during consecutive 15-s intervals and the mean data for each treatment group. The data were visually inspected using the SleepWatch program (MiniMitter, Sunriver, OR) and imported into StatView (SAS Institute, Inc., NC) for analysis.

2.3. Rest deprivation

Fish were transferred to constant darkness and the basal recording was conducted on Day 1. On Day 2, fish were subject to continuous vibration (4 mm amplitude, 1 Hz), either during subjective day (ZT0–ZT6) or during subjective night (ZT18–ZT24). The control group was maintained in similar conditions without experiencing vibration.

2.4. Arousal threshold

Arousal threshold was measured in constant darkness during subjective day (ZT3–5) or subjective night (ZT15–17). At regular 20-min intervals, four series of six mechanical stimuli each (gentle standardized tapping every 2 s) were applied to a microplate containing fish. The video recordings of this procedure were visually scored and a number of stimuli that induced locomotion in individual fish was registered as arousal threshold scores 1–6, with the score 7 assigned to a non-responding individual. Mean scores of four series were used for further analysis.

2.5. Pharmacological treatments

Treatment and control groups were placed in successive rows of a microplate, with each well containing 350 μl of water, and were recorded continuously starting at ZT 3. Following a 2-h basal recording, a treatment solution (50 μl), containing melatonin, diazepam or pentobarbital, was administered directly into individual wells containing larvae and the recording was resumed for 2 more hours. Although the onset of the behavioral effects typically occurred within 20 min after treatment (as discussed below), a 2-h period of recording was found to be optimal for the dose-dependency studies. Flumazenil (1 μM) or luzindole (10 μM) was administered 20 min prior to treatment with 100 nM of pentobarbital, diazepam or melatonin, or control solutions (vehicle). The doses listed reflect final concentrations of these agents in the wells of a microplate.

To prepare stock solutions, melatonin (Nestle, Switzerland) and luzindole (Sigma, St. Louis, MO) were dissolved in ethanol to 100 mM and 10 mM, correspondingly. Sodium pentobarbital (Sigma, St. Louis, MO) and flumazenil (Roche, Nutley, NJ) were dissolved in water. Diazepam stock solution (5 mg/ml; Elkins-Sinn, Inc., Cherry Hill, NJ) contained 40% propylene glycol, 10% ethyl alcohol, 5% sodium benzoate and 1.5% benzyl alcohol. All stock solutions were diluted to final concentrations with water. The control for each treatment concentration contained corresponding vehicle solutions.

2.6. Statistical analysis

Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), followed by post-hoc comparisons between the experimental groups using Tukey' test, with level of significance set at $P<0.05$ (StatView software; SAS Institute, Inc., NC). Data are presented as mean±S.E.M.

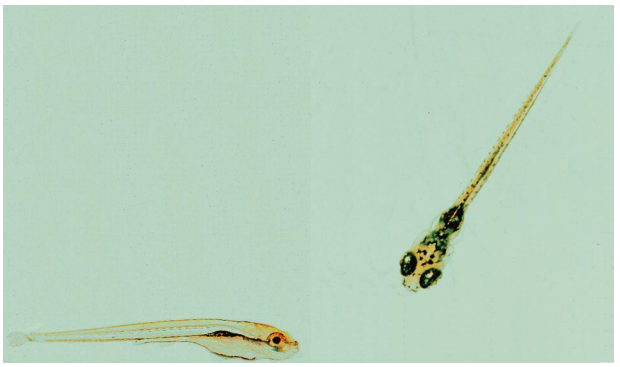

Fig. 1. Typical rest postures in larval zebrafish: floating with head down or staying horizontal, close to the bottom of a chamber.

3. Results

Visual observations in larval zebrafish, conducted during the day or at night (using IR illumination), showed that prolonged periods of immobility (over 10 min) in larval zebrafish, occurring predominantly at night, were typically associated with two main postures, either floating with head down or staying in a horizontal position close to the bottom of a chamber (Fig. 1).

A continuous (up to 5-day) monitoring showed that the circadian variation in zebrafish locomotor activity, with higher levels during subjective day and lower levels at subjective night, was preserved after fish were transferred from a 12:12 h light–dark cycle to constant darkness (Fig. 2a,b). A nocturnal decline in motor activity was accompanied by a significant increase in arousal threshold relative to daytime (Fig. 2c; $P<0.01$). These results confirm that the rest state in larval zebrafish is under control of the intrinsic circadian system [2].

Daytime rest deprivation did not significantly affect rest behavior thereafter, though some decrease in daytime locomotor activity (Fig. 2 b; $P<0.05$) and elevation in the arousal threshold was noticed (Fig. 2 d). In contrast, nighttime rest deprivation resulted in a significant decline in daytime locomotor activity (Fig. 2a; $P<0.01$) and in a heightened arousal threshold (Fig. 2d; $P<0.01$), compared to basal recordings. These data suggest that the vibration procedure used does not significantly alter animals' behavior and that the rest state in zebrafish is under homeostatic control.

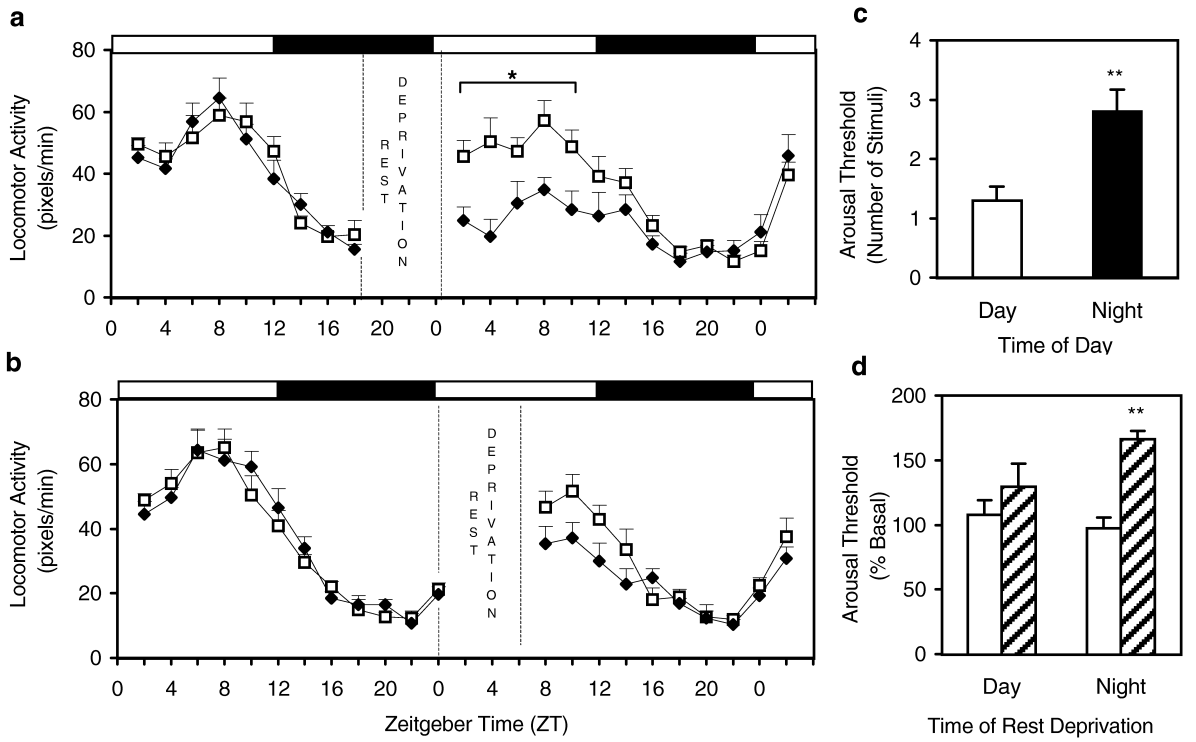

Fig. 2. Daily variation in locomotor activity and arousal threshold in larval zebrafish maintained in constant darkness and a compensatory reduction in locomotor activity and increase in arousal threshold following rest deprivation. (a, b) Zeitgeber time (ZT) and horizontal white/black bars indicate subjective day versus subjective night, according to 12:12 light–dark cycle prior to the beginning of recording, with ZT0 corresponding to lights on time. Each data point represents mean±S.E.M. group locomotor activity for preceding 2 h of recording (pixels per minute). $N=60$ for each group. The rest deprivation was scheduled either (a) during subjective night (ZT18–ZT24), or (b) during subjective day (ZT0–ZT6). Closed diamonds – rest deprivation group, open squares – control group. (c) Arousal threshold was measured in constant darkness during subjective day (ZT3–5) or subjective night (ZT15–17); $N=20$ for each group. (d) Changes in daytime arousal threshold (% of Basal) starting an hour after daytime or nighttime rest deprivation. White bars – control; striped bars – rest deprivation. $N=20$ for each group. *<0.05; **$P<0.01$.

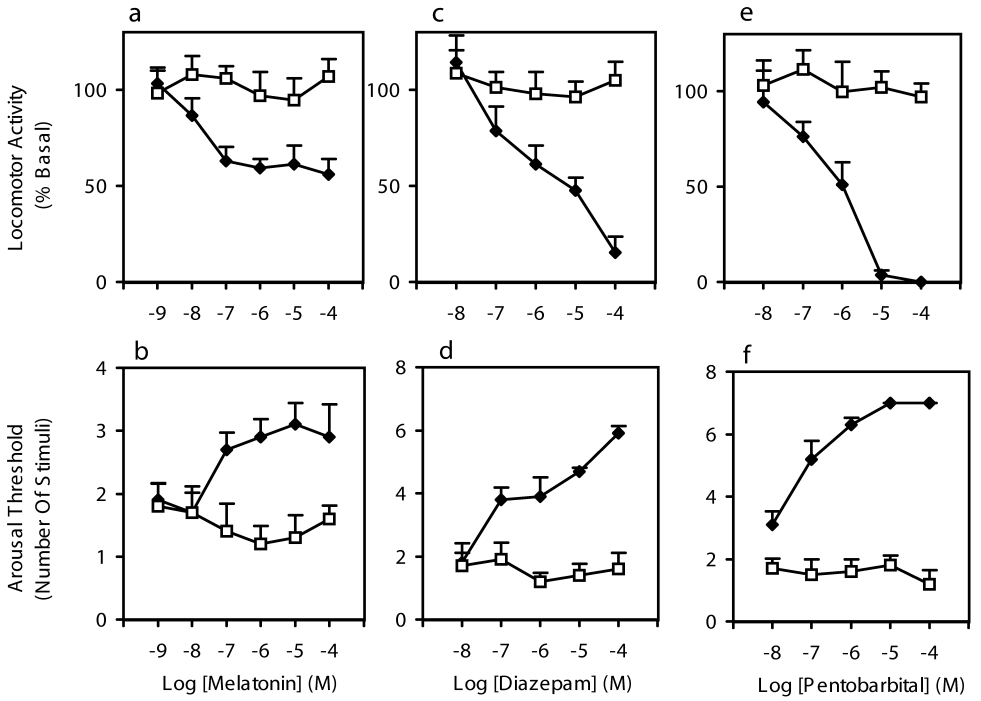

Fig. 3. Melatonin and conventional sedatives promote rest behavior in larval zebrafish. Melatonin, diazepam and sodium pentobarbital (barbital) significantly and dose-dependently reduced zebrafish locomotor activity (a, c, e) and increased arousal threshold (b, d, f). Each data point represents mean±S.E.M. group changes in a 2-h locomotor activity relative to basal activity, measured in each treatment or control group for 2 h prior to treatment administration. Arousal threshold data are expressed as the mean±S.E.M. group number of stimuli necessary to initiate locomotion in a resting fish. Closed diamond – treatment, open square – vehicle control; $N=20$, each group.

Collectively, the presence of characteristic postures, elevated arousal threshold to sensory stimulation during habitual nighttime hours of prolonged rest and a compensatory rest rebound following rest deprivation allowed us to classify the rest state in larval zebrafish as a sleep-like state, as we will refer to it below.

The exposure to a wide range of melatonin concentrations (10 nM–100 μM) promoted a sleep-like state in zebrafish, reducing their locomotor activity and elevating their arousal threshold within 20 min after the treatment (Fig. 3a,b; $P<0.01$). The pretreatment with specific melatonin receptor antagonist, luzindole [6], blocked melatonin-induced reduction in locomotor activity (Fig. 4a). In contrast, the specific benzodiazepine receptor antagonist, flumazenil, did not modify this effect of melatonin (Fig. 4a). Neither flumazenil nor luzindole was able to significantly alter daytime locomotor activity in zebrafish when administered alone. These results indicate

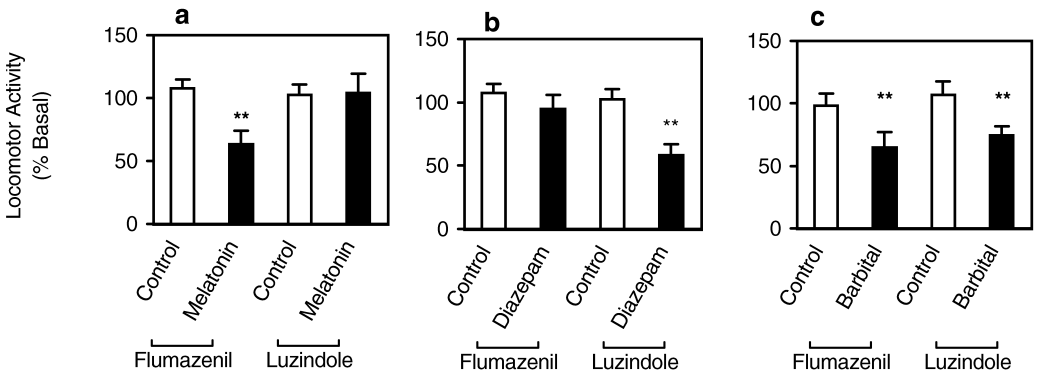

Fig. 4. Melatonin and diazepam affect locomotor activity in zebrafish via specific membrane receptors. Pretreatment with specific antagonist for melatonin receptors, luzindole, blocked the decline in locomotor activity induced by (a) melatonin, but not by (b) diazepam or (c) pentobarbital. Pretreatment with specific benzodiazepine receptor antagonist, flumazenil, blocked reduction in locomotor activity following (b) diazepam, but not (a) melatonin or (c) pentobarbital treatment. Control solutions are vehicles for each treatment used. Data are expressed as mean±S.E.M. group changes (%) in daytime locomotor activity, measured for 2 h after treatment, relative to basal activity. $N=30$, each group; $^{**}P<0.01$.

that melatonin promotes sleep-like state in larval zebrafish and this effect is mediated via specific melatonin receptor(s).

Administration of pentobarbital (10 nM–100 μM) resulted in a concentration-dependent increase in sleep-like behavior in zebrafish ($P<0.01$). Lower concentrations of pentobarbital reduced daytime locomotor activity to within typical nighttime levels, while higher concentrations eliminated spontaneous locomotion (Fig. 3e). Besides, this barbiturate either significantly increased the arousal threshold or abolished larval response to external stimuli (Fig. 3f). Similarly, diazepam (10 nM–100 μM), in a concentration-dependent manner, reduced larval locomotor activity (Fig. 3c; $P<0.01$) and increased arousal threshold to levels within or above those observed during normal nighttime rest (Fig. 3d; $P<0.01$). Flumazenil blocked changes in locomotor activity induced by diazepam (Fig. 4b) but not affected pentobarbital-induced reduction in locomotor activity (Fig. 4c). Specific melatonin receptor antagonist, luzindole, did not alter the effects of either diazepam or pentobarbital (Fig. 4b, c). These data suggest that the sleep-like state in zebrafish is sensitive to the modulation of GABA-ergic pathway by benzodiazepine and barbiturate hypnotics.

4. Discussion

The data accumulated in this study show that the rest state in zebrafish has fundamental similarities with behavioral manifestations of sleep in mammals, including characteristic postures and temporary reduction in sensitivity to sensory inputs. Furthermore, the rest behavior in zebrafish is regulated by the circadian system, since periodic reduction in locomotor activity and the increase in arousal threshold are maintained in constant darkness and occur during subjective night. Analogous to sleep in mammals, zebrafish show a compensatory rest rebound, reducing locomotor activity and increasing the arousal threshold following rest deprivation. This allows suggesting that zebrafish have a homeostatic control of rest behavior. In addition, the conventional hypnotic agents of benzodiazepine and barbiturate families potentiate the rest behavior in zebrafish. Collectively, these results indicate that the rest behavior in larval zebrafish can be considered a sleep-like state.

The primary goal of this study was to develop a simple, genetically tractable diurnal vertebrate model for studying the mechanisms of melatonin action on sleep. Indeed, we found larval zebrafish to be highly sensitive to the sleep-promoting effect of melatonin, with both spontaneous locomotor activity and the arousal threshold in resting fish being affected by the pineal hormone. Remarkably, after either low or high doses of melatonin were administered, the reduced locomotor activity and the increased arousal threshold remained within the range normally observed in zebrafish at night. Furthermore, when the behavioral arousal was achieved with sensory stimulation, animals treated with melatonin showed vigorous locomotion, suggesting that the hormone did not alter the animals' ability to perform, but rather reduced their sensitivity to sensory stimuli. It should be noted that earlier observations in humans also showed that a wide range of melatonin doses administered at daytime could significantly promote sleep onset, without producing an imperative drive for sleep or deep sedation, and with little difference in the effects of physiological and pharmacological doses [5,14,21].

In contrast to melatonin treatment, the effects of both diazepam and pentobarbital showed a pronounced dose-dependency, with high doses promoting a reduction in spontaneous or evoked locomotor activity well beyond the levels observed in zebrafish during normal rest period. The behavioral effects of benzodiazepines and barbiturates in humans and other mammals are also known to be highly dose dependent and can cause from mild sleep-promoting effect to the loss of consciousness.

Importantly, this study provides the first evidence that the effect of melatonin on sleep is mediated through specific melatonin receptors. The high-affinity Gi-protein-coupled receptors for melatonin were identified in different species [12]. Their location in sensory and integrative areas of the brain in mammals and birds, as well as within the site of the major circadian pacemaker, suggest their link to the behavioral manifestations of increased melatonin levels [19]. In zebrafish, five different melatonin receptor fragments were cloned, representing a melatonin receptor family consisting of three distinct subtypes found in other vertebrate species [13]. The localization of these receptors in zebrafish is not yet known.

We studied the effects of pretreatment with specific melatonin receptor antagonist, luzindole, on the sleep-promoting effects of melatonin, diazepam and pentobarbital in zebrafish, comparing them to the effects of specific benzodiazepine receptor antagonist, flumazenil. Luzindole selectively blocked the effect of melatonin, but did not change the effects of diazepam and pentobarbital, while flumazenil was able to counteract only the effect of diazepam. Thus, the effects of melatonin on sleep-like state in zebrafish are mediated via specific melatonin receptors, rather than through benzodiazepine receptors. To further explore which of several melatonin receptors mediate the effect of the pineal hormone on sleep, would require using selective analogs for individual melatonin receptors, not available so far, or exploiting zebrafish mutants lacking functional copies of a particular melatonin receptor.

In summary, our data suggest that the pineal hormone melatonin is part of the evolutionary conserved neuroendocrine pathway of sleep regulation in diurnal vertebrates and its effect on sleep is mediated through specific melatonin receptors. Furthermore, the behavioral, endocrine and pharmacological similarities between sleep-like state in zebrafish and human sleep, and recent advances in

studying zebrafish genetics and development [15,18], provide a unique opportunity for genetic examination of the mechanisms of sleep regulation in vertebrates and could help to develop new strategies for treating sleep disorders in humans.

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