Chapter 2

Melatonin is required for the circadian regulation of sleep

Abstract

Sleep is an evolutionarily conserved behavioral state whose regulation is poorly understood. A classical model posits that sleep is regulated by homeostatic and circadian mechanisms. Several factors have been implicated in mediating the homeostatic regulation of sleep, but molecules underlying the circadian mechanism are unknown. Here we use animals lacking melatonin due to mutation of arylalkylamine N-acetyltransferase 2 (*aanat2*) to show that melatonin is required for circadian regulation of sleep in zebrafish. Sleep is dramatically reduced at night in *aanat2* mutants maintained in light/dark conditions, and the circadian regulation of sleep is abolished in free-running conditions. We find that melatonin promotes sleep downstream of the circadian clock as it is not required to initiate or maintain circadian rhythms. Additionally, we provide evidence that melatonin may induce sleep in part by promoting adenosine signaling, thus potentially linking circadian and homeostatic control of sleep.

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Introduction

Sleep is an evolutionarily conserved behavioral state whose regulation is poorly understood (Sehgal and Mignot, 2011). A key model postulates regulation by a homeostatic process that responds to internal cues for sleep need and a circadian process that responds to external cues (Borbély, 1982). Several lines of evidence suggest that accumulation of extracellular adenosine in specific brain regions plays an important role in the homeostatic mechanism, although it is clear that other factors are also required (reviewed in Brown et al., 2012). However, while mechanisms that regulate the circadian clock are well characterized (Fisher et al., 2013), molecules that transmit circadian information to regulate sleep are largely unknown.

Melatonin is an attractive candidate for mediating the circadian process because the clock regulates its production (Klein, 2007) and it induces sleep in some contexts (Fisher et al., 2013). However, despite decades of study and widespread use, the role of melatonin in regulating sleep is controversial (Fisher et al., 2013). Exogenous melatonin is reported to have sleep-promoting effects in diurnal vertebrates including humans (Brzezinski et al., 2005; Zhdanova, 2005), nonhuman primates (Zhdanova et al., 2002), domesticated cats (Goldstein and Pavel, 1981), birds (Mintz et al., 1998), and zebrafish (Zhdanova et al., 2001). However, others failed to observe this effect (Buscemi et al., 2006; van den Heuvel et al., 2005), and its endogenous role in any animal is unknown. Exogenous melatonin can entrain (Lockley et al., 2000; Sack et al., 2000) and phase shift (Lewy et al., 1992) the circadian clock in some contexts, and melatonin has been called a regulator of circadian rhythms (Elbaz et al., 2013). Indeed, melatonin has been proposed to promote sleep indirectly by phase advancing the circadian clock (Arendt, 2003) or by

inhibiting the circadian drive for wakefulness (Scheer and Czeisler, 2005). However, these hypotheses are based on exogenous melatonin. Determining melatonin's role in sleep is complicated by its production at night in both diurnal and nocturnal animals, and because most laboratory mouse strains produce little or no melatonin (Goto et al., 1989). As a result, studies have relied on pharmacological approaches that have produced inconsistent results (Fisher et al., 2013). To determine whether endogenous melatonin regulates sleep in a diurnal vertebrate, we analyzed melatonin function in the zebrafish, whose circadian clock and sleep regulation are conserved with mammals (Elbaz et al., 2013).

Results

Melatonin promotes initiation and maintenance of night-time sleep

Melatonin is produced in the zebrafish pineal gland at night under control of the circadian clock (Kazimi and Cahill, 1999), as in mammals. To determine whether endogenous melatonin is required for sleep, we generated zebrafish with a predicted null mutation in *arylalkylamine N-acetyltransferase 2* (*aanat2*) (Figure 2.2A), which is required for melatonin synthesis in the pineal gland (Klein, 2007). We found that wild-type (WT) larvae in 14:10 hr light:dark (LD) conditions had high melatonin levels at night and low levels during the day, while *aanat2* homozygous mutants *(aanat2*$^{-/-}$) produced little to no melatonin (Figure 2.2B). To determine whether melatonin is required for sleep, we used a videotracking assay (Prober et al., 2006) to compare sleep/ wake behaviors of *aanat2*$^{-/-}$ larvae to their *aanat2*$^{+/-}$ and WT siblings. We found that all three genotypes exhibited similar day- time amounts of sleep and activity (Figures 2.1A–C and E). However, at night, *aanat2*$^{-/-}$ larvae slept almost half as much and were three times more active than controls (Figures 2.1A,B, D, F). Decreased nighttime sleep was due mainly to a decrease in sleep bout length and a corresponding increase in wake bout length, with little effect on bout number (Figure 2.3B,D,H). Sleep latency (time between lights-out and sleep) at night was also longer for aanat2$^{-/-}$ larvae (Figure 2.3J). These results demonstrate that endogenous melatonin promotes initiation and maintenance of nighttime sleep in a diurnal vertebrate.

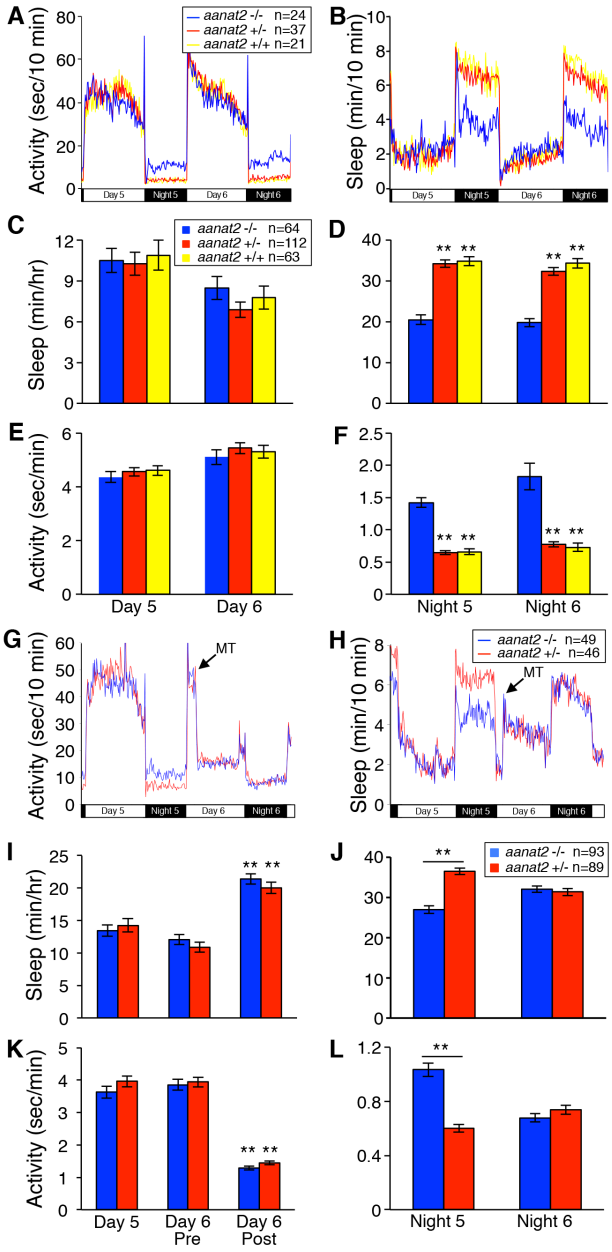

Figure 2.1 *aanat2-/-* larvae sleep less at night. (A-F) *aanat2-/-* larvae sleep less (B,D) and are more active (A,F) than sibling controls at night, but not during the day (A,B,C,E). (G-L) Arrow indicates addition of 10μM melatonin on day 6. *aanat2-/-* sleep and locomotor activity phenotypes of night 5 are absent on night 6 (G,H,J,L). During the day, exogenous melatonin decreases locomotor activity (G,K) and increases sleep (H,I) for both genotypes. Day 6 Pre and Post refer to periods before and after melatonin addition. Total sleep amount can vary for nights 5 and 6 of development, so comparisons between different genotypes should be made on the same night. Data are from one representative experiment (A-B,G-H) or combined from two (I-L) or three (C-F) experiments. Bar graphs represent mean ± SEM. n, number of larvae. **, $p<0.01$ compared to aanat2-/- (C-F), each genotype Day 6 Post compared to Day 6 Pre (I,K), or the indicated comparisons (J,L) by Dunnett's (C-F) or Tukey's test (I-L). See also Figure 2.3.

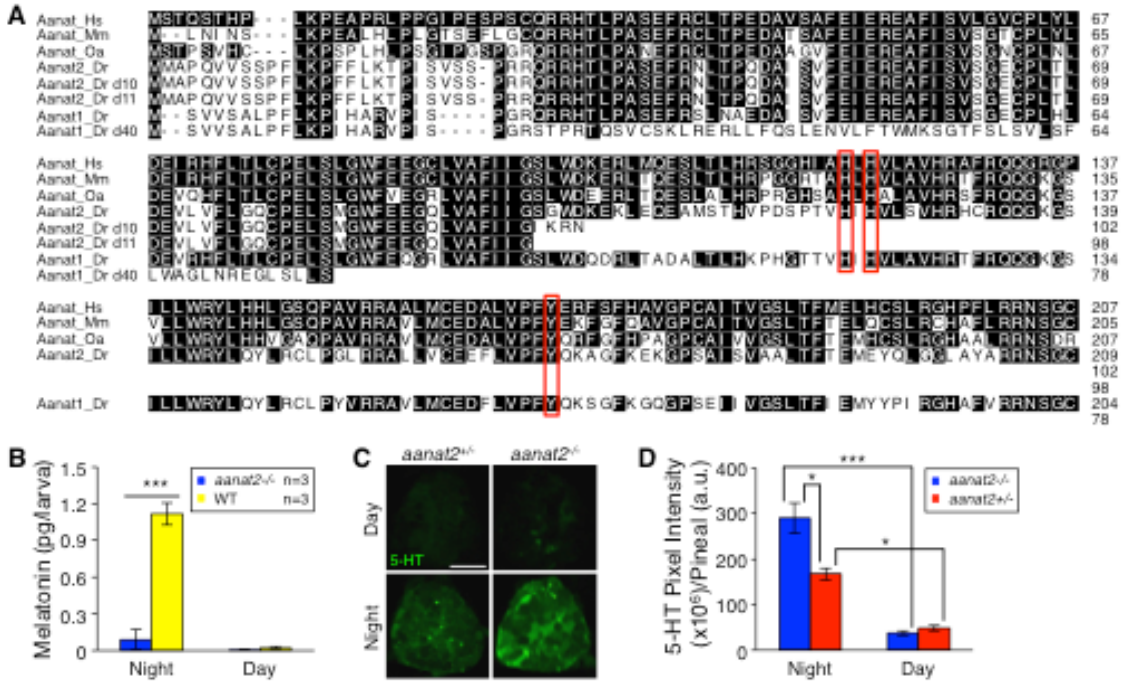

Figure 2.2 *aanat* mutant sequences and effects *aanat2* mutation on melatonin and serotonin levels (A) Amino acid sequence alignment of human (Hs), mouse (Mm) and sheep (Oa) Aanat, and zebrafish (Dr) Aanat1 and Aanat2, are shown. The sequences of two zebrafish Aanat2 mutant proteins (d10 and d11) and one zebrafish Aanat1 mutant protein (d40) that were generated in this study are also shown. Red boxes indicate conserved amino acids that interact with the acetyl-CoA substrate and are critical for enzyme activity in the sheep Aanat protein (Hickman et al.,1999; Scheibner et al., 2002). (B) *aanat2-/-* larvae contain little or no melatonin. Melatonin concentration was assayed by ELISA from WT and *aanat2-/-* larvae at the end of the night (6 am) or end of the day (6 pm). Little or no melatonin was detected in WT larvae at 6 pm or in *aanat2-/-* larvae at either time point. The assay was performed in triplicate, with 5 larvae per sample. Mean ± SEM is shown. ***, p<1x10-6 by Student's t-test. (C, D) Serotonin (5-HT) levels in the pineal gland are higher at night compared to the day in both *aanat2+/-* and *aanat2-/-* larvae, and are higher at night in *aanat2-/-* larvae compared to their *aanat2+/-* siblings. Single 0.75 µm thick confocal sections are shown. Number of animals analyzed: *aanat2+/-* day=11, *aanat2+/-* night=6, *aanat2-/-* day=7, *aanat2-/-* night=13. Mean ± SEM is shown. *, p<0.05. ***, p<1x10-6 By Tukey's test. Scale=20 µm.

Aanat2 loss-of function phenotype is mediated by melatonin and not serotonin

Serotonin is acetylated by AANAT to form acetylserotonin, which is methylated to form melatonin. Loss of aanat2 may thus elevate serotonin levels in the pineal gland. Consistent with mammalian results (Borjigin et al., 2012), we found that serotonin levels are higher in the pineal. Serotonin levels were even higher at night in *aanat2*$^{-/-}$ larvae compared to their *aanat2*$^{+/-}$ siblings, indicating that loss of *aanat2* results in higher pineal serotonin levels (Figure 2.2 C,D). To test whether the *aanat2*$^{-/-}$ behavioral phenotype is due to loss of melatonin or increased serotonin, we added exogenous melatonin, which completely rescued the aanat2$^{-/-}$ phenotype (Figure 2.1 G-L), indicating that it is due to loss of melatonin.

Aanat1 is not required for sleep

Zebrafish have a second aanat ortholog (*aanat1*) that is expressed in the retina (Appelbaum et al., 2006). To test whether aanat1 is partially redundant with aanat2 in regulating sleep, we generated zebrafish with a predicted null mutation in aanat1 (Figure 2.2A). The behavioral phenotype of *aanat1*$^{-/-}$*; aanat2*$^{-/-}$ larvae was indistinguishable from that of *aanat2*$^{-/-}$ larvae (data not shown), suggesting that aanat1 is not required for sleep.

Aanat2 mutants exhibit normal sensory responsiveness/arousal

Since *aanat2*$^{-/-}$ larvae sleep less at night, we hypothesized that their arousal threshold might be reduced. To test this hypothesis, we applied a mechanoacoustic stimulus (Woods et al., 2014) at night at 1-minute intervals at a range of intensities. Surprisingly, we found that *aanat2*$^{-/-}$larvae and their sibling controls exhibited similar

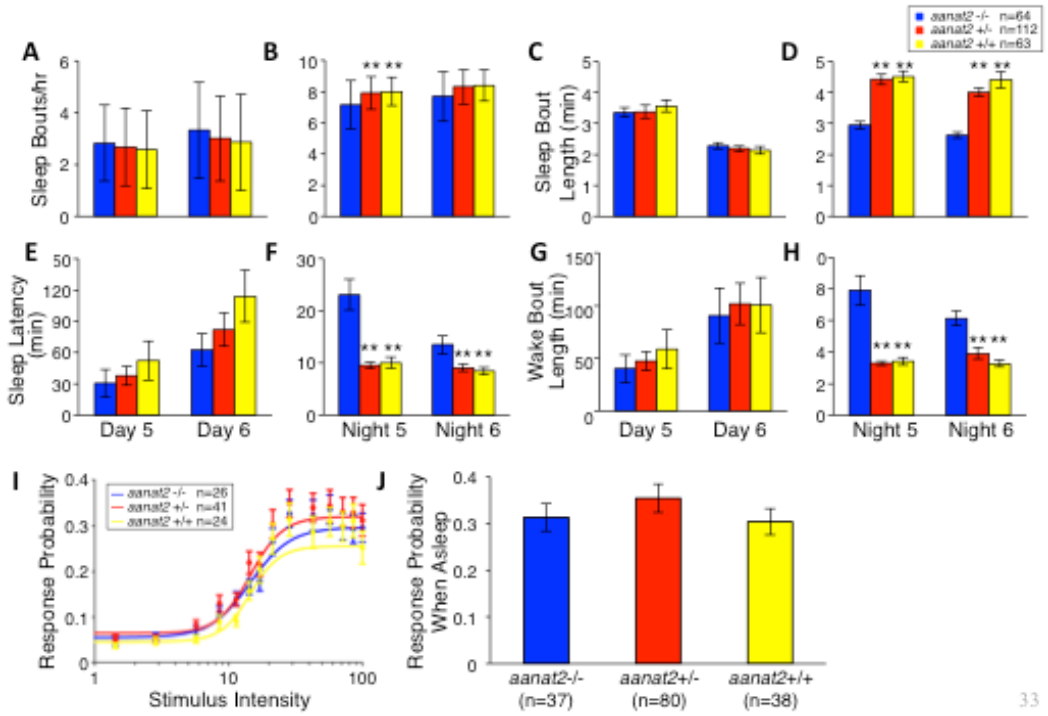

Figure 2.3 Effects *aanat2* mutation on behavior in LD and sensory responsiveness/arousal (Related to Figure 2.1). (A-H) *aanat2-/-* larvae are more active and sleep less at night in LD compared to their heterozygous mutant and WT (siblings. Graphs are based on the data shown in Figures 1A-1F. *aanat2-/-* larvae exhibit slightly fewer sleep bouts (B), shorter sleep bouts (D), longer sleep latency (F), and longer wake bouts (H) at night, but no phenotype during the day (A, C, E, G). Data is combined from three experiments. Median ± median absolute deviation (MAD) (A, B) and mean ± SEM (C-H) are shown. n, number of larvae analyzed. *, $p<0.05$; **, $p<0.01$ compared to *aanat2-/-* by Steel-Dwass (A, B) or Dunnett's test (C-H). (I) The response probability to a mechanoacoustic stimulus applied once per minute at night over a range of stimulus intensities is similar for all genotypes (log (half-maximal response probability) = 1.17±0.03, 1.15±0.03 and 1.16±0.04 for *aanat2-/-, aanat2+/-* and *aanat2+/+,* respectively; $p=0.92$ by extra sum-of-squares F test). Each data point represents the mean response probability ± SEM at a particular stimulus intensity. The x- axis shows 0% to 100% stimulus intensity plotted as arbitrary units on a logarithmic scale. (J) When taps were applied every 5 minutes, sleeping (inactive for 1 or more minutes) *aanat2-/-*, *aanat2+/-* and *aanat2+/+* larvae were equally likely to respond (response probability = 0.31±0.03, 0.35±0.03, and 0.30±0.03, respectively; $p=0.43$ by one-way ANOVA). Mean ± SEM is shown. n, number of larvae analyzed.

half-maximal response probabilities (log(probability) 1.17±0.03, 1.15±0.03 and 1.16±0.04 for *aanat2*$^{-/-}$, *aanat2*$^{+/-}$ and *aanat2*$^{+/+}$, respectively; p=0.92 by extra sum-of-squares F test) (Figure 2.3I), indicating that aanat2$^{-/-}$ larvae have a normal arousal threshold. We next asked whether sleeping *aanat2*$^{-/-}$ larvae are more likely to awaken in response to a stimulus, since light sleep is often a feature of insomnia. To test this hypothesis, we stimulated larvae every 5 minutes, allowing larvae to re-enter sleep after each stimulus. We used a tap strength at which 50% of larvae normally respond. There was no significant difference among the three genotypes (p=0.43 by one-way ANOVA) (Figure 2.3J). Thus, although aanat2$^{-/-}$ larvae sleep less at night, their arousal threshold and sleep depth are similar to controls, suggesting that their sleep defect is not caused by hyperarousal.

Pineal ablation results in increased activity and reduced sleep at night

As an alternative approach to deplete melatonin we ablated melatonin-producing cells. We generated transgenic zebrafish in which the *aanat2* promoter drives expression of cyan fluorescent protein fused to nitroreductase (CFP-NTR), a bacterial protein that converts the inert pro-drug metronidazole (MTZ) into a potent DNA cross-linking agent that causes cell- autonomous death (Curado et al., 2007). CFP-NTR was expressed exclusively in aanat2- expressing pineal gland cells (Figure 2.4A, B), which developed normally but underwent cell death upon addition of MTZ (Figure 2.4C-E). Some pineal gland cells died within a few hours of adding MTZ, but we observed the most robust ablation without non-specific drug toxicity with treatment from 60-80 and 108-128 hpf,

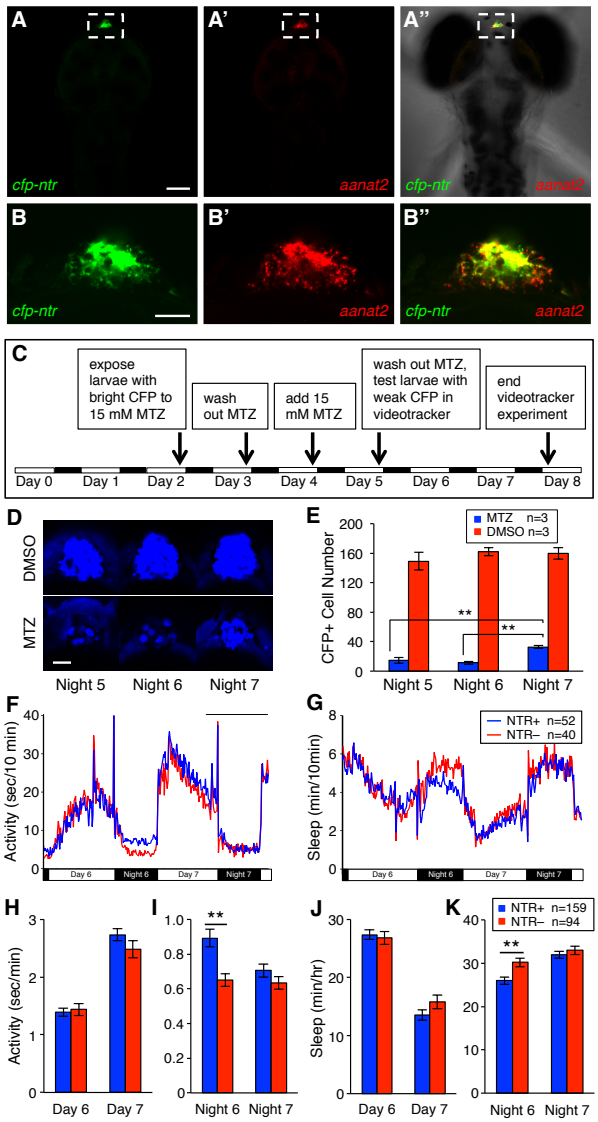

Figure 2.4. Depletion of aanat2-expressing pineal gland cells decreases sleep at night (Related to Figure 2.1). (A, B) Double fluorescent in situ hybridization using probes specific for cfp-ntr (green) and aanat2 (red) was performed on a Tg(*aanat2*:CFP-NTR) larva fixed at 120 hpf. Boxed region in (A) is shown at higher magnification in (B). Scale bar=100 μm (A) and 20 μm (B). cfp-ntr expression is only observed in aanat2-expressing pineal gland cells. (C) Ablation experiment outline. Larvae were treated with 15 mM MTZ from 60-80 and 108-128 hpf, and behavioral monitoring was initiated at 134 hpf. (D) aanat2:CFP-NTR expressing cells in the pineal gland were imaged during the fifth, sixth and seventh nights of development in larvae treated with DMSO vehicle control or MTZ. Scale bar=20 μm. (E) Quantification of *aanat2*:CFP-NTR cells. Mean ± SEM is shown. n, number of larvae quantified. (F-K) Tg(*aanat2*:CFP-NTR) larvae exhibit increased locomotor activity (F, I) and decreased sleep (G, K) during night 6 compared to non-transgenic siblings. The phenotype is absent on night 7, presumably due to the increased number of aanat2-expressing cells (D, E). Data are from one representative experiment (F,G) or 3 experiments combined (H-K). **, $p<0.01$ for the indicated comparisons by Tukey's test.

removing MTZ between treatments (Figure 2.4C-E). As observed for *aanat2*$^{-/-}$ larvae, *aanat2*-CFP-NTR larvae slept less and were more active at night (Figure 2.4F-K), although the effect was weaker, likely due to persistence of a small number of aanat2-expressing cells (Figure 2.4D,E). Consistent with this hypothesis, 48 hours after MTZ removal we observed a small number of new aanat2-expressing cells (Figure 2.4D, E) and larvae exhibited normal amounts of sleep and activity (Figure 3F-K), indicating that the aanat2-CFP- NTR phenotype is reversible and suggesting that a small number of pineal gland cells are sufficient for normal sleep.

Endogenous melatonin promotes sleep directly rather than via the circadian clock

Melatonin has been proposed to promote sleep indirectly, by phase-advancing the circadian clock (Arendt, 2003) or inhibiting the circadian drive for wakefulness (Scheer and Czeisler, 2005). To determine whether the sleep-promoting role of endogenous melatonin results from effects on the circadian clock, we raised and tested *aanat2*$^{-/-}$ larvae in the dark (DD), resulting in arrhythmic animals lacking overt behavioral or molecular circadian rhythms (Figures 2.5A-F and 2.6G-H) (Kaneko and Cahill, 2005). To determine whether the arrhythmia observed in DD is due to arrested or asynchronous cellular circadian clocks (Dekens and Whitmore, 2008) we performed fluorescent in situ hybridization for the clock gene per1b (Figure 2.6I-Q). If the lack of overt rhythms is caused by arrested clocks, per1b should be expressed similarly at all circadian time points. However, if it results from asynchronous clocks, expression should be more heterogeneous in larvae raised in DD than in those raised in LD. Furthermore, for asynchronous clocks, the average fluorescence intensity among many cells in DD should

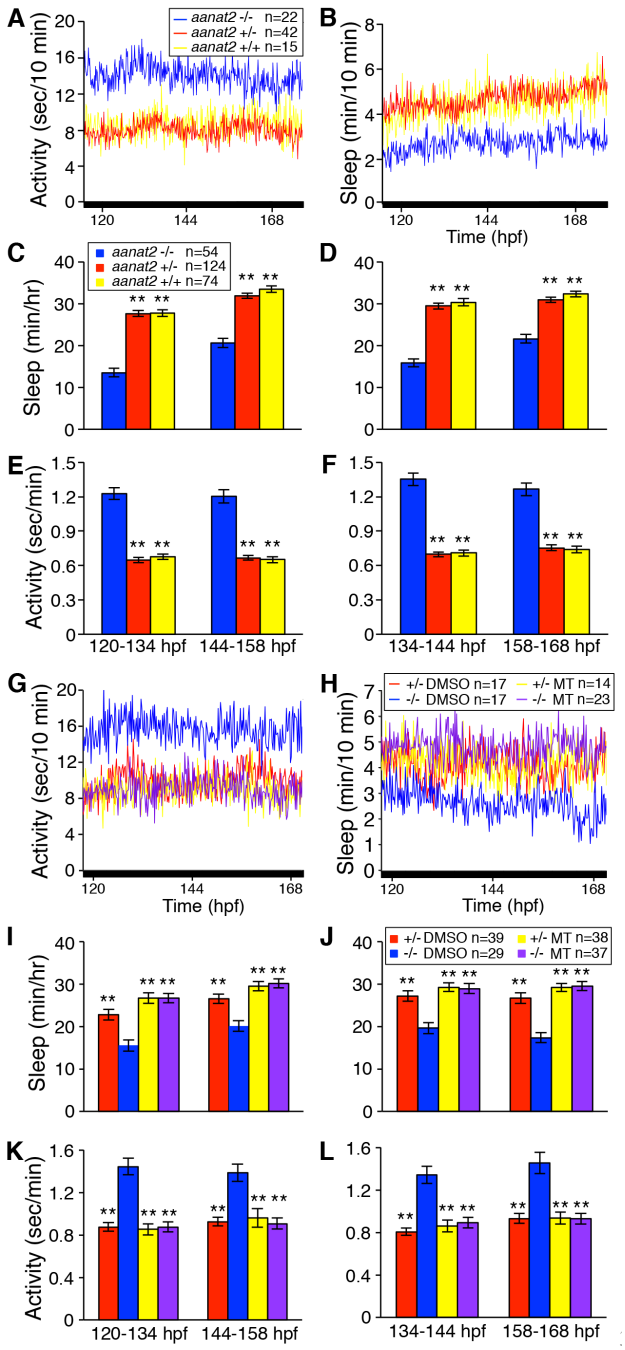

Figure 2.5. Reduced sleep in *aanat2-/-* zebrafish does not require an entrained circadian rhythm. (A,B) Larvae raised and tested in DD do not exhibit circadian locomotor activity or sleep rhythms. *aanat2-/-* larvae are more active (A,E,F) and sleep less (B-D) than sibling controls. (G-L) *aanat2-/-* larvae treated with vehicle control are more active (G,K,L) and sleep less (H-J) than *aanat+/-* siblings. *aanat2-/-* larvae treated with 10 μM melatonin exhibit locomotor activity and sleep indistinguishable from their melatonin-treated *aanat+/-* siblings (G-L). Data are from one representative experiment (A-B,G-H), or combined from two (I-L) or three (C-F) experiments. Bar graphs represent mean ± SEM for the 5th and 6th days and nights of development. **, $p<0.01$ compared to *aanat2-/-* (C-F) or *aanat2-/-* DMSO (I-L) by Dunnett's test. See also Figure 2.6

be lower than the peak level in LD. We found that per1b expression oscillates throughout the brain in LD (Figure 2.6I, K, M, O) with phasing consistent with data from reverse-transcription quantitative PCR (RT-qPCR) (Figure 2.6G). In contrast, per1b is expressed at a similar level at all circadian time points in larvae raised in DD (Figure 2.6J, L, N, P). Quantification of fluorescence intensity in the hypothalamus showed that average per1b levels in DD are similar to peak levels in LD (Figure 2.6Q). Quantification in the forebrain and midbrain produced similar results (data not shown). These results suggest that raising larvae in DD abolishes circadian rhythms at the cellular level. Under these conditions, *aanat2*$^{-/-}$ larvae exhibited continuously decreased sleep and increased activity (Figure 2.5A-F). The magnitude of the difference was similar to that observed at night in LD (Figure 2.1A-F), and was again due to a decrease in sleep bout length and corresponding increase in wake bout length (Figure 2.6A-F). This phenotype was also rescued by exogenous melatonin (Figure 2.5G-L). These results suggest that endogenous melatonin promotes sleep directly rather than via the circadian clock in zebrafish.

Melatonin is not required for behavioral or molecular circadian rhythms

Having established that melatonin is required for sleep at night, we next asked whether melatonin is required for normal circadian rhythms. Studies in humans, nonhuman primates and rodents have shown that exogenous melatonin can phase-shift (Lewy et al., 1992) and entrain (Lockley et al., 2000; Sack et al., 2000) the circadian clock. However, loss of the pineal gland, and thus melatonin production, produces inconsistent results (Arendt, 2003). While pinealectomy in some species of fish, birds and reptiles affects circadian rhythms (Underwood, 2001), it is unclear whether this is due to

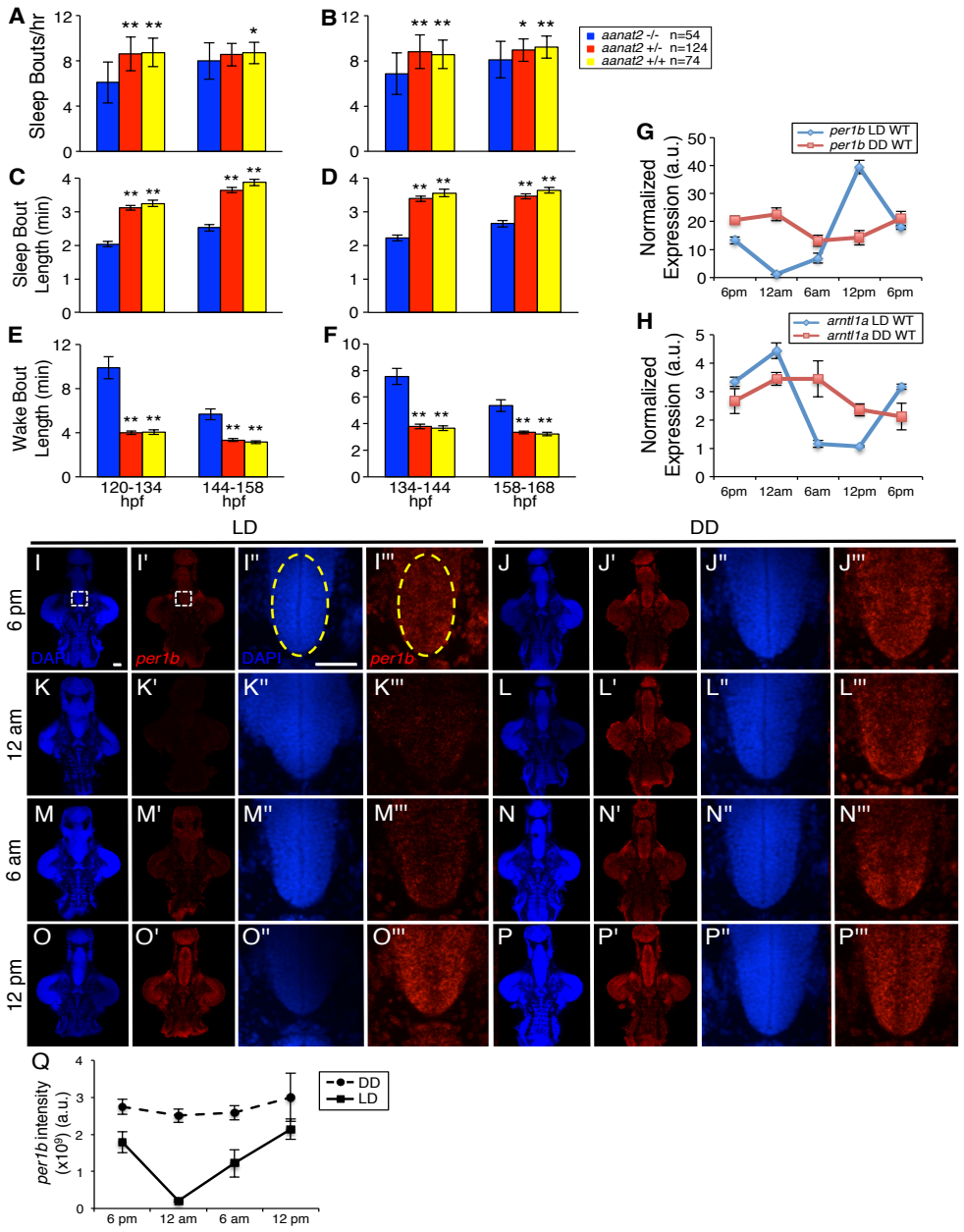

Figure 2.6. Reduced sleep in *aanat2-/-* zebrafish does not require an entrained circadian rhythm (Related to Figure 2.5). (A-F) Graphs are based on data shown in Figures 2A-2F. When raised and tested in DD, *aanat2-/-* larvae exhibit fewer sleep bouts (A, B), shorter sleep bouts (C, D) and longer wake bouts (E, F) during periods corresponding to the 5th and 6th days and nights of development. Data are combined from three experiments. Median ± MAD (A, B) and Mean ± SEM (C-F) are shown. n, number of larvae analyzed. *, $p<0.05$; **, $p<0.01$ compared to *aanat2- /-* by Steel-Dwass (A, B) or Dunnett's test (C-F). (G, H) WT larvae raised in DD (red) exhibit dramatically reduced or absent rhythmic expression of the circadian clock genes *per1b* (G) and *arntl1a* (H) compared to siblings raised in LD (blue). Samples were collected at 6-hour intervals from 6 pm on day 5 until 6 pm on day 6. The expression of both mRNAs display robust oscillations in LD but not in DD (peak:trough ratio is 33 in LD versus 2 in DD for *per1b*,

and 4.2 in LD versus 1.6 in DD for *arntl1a*). None of the DD data points for *per1b* or *arntl1a* is significantly different from any other ($p>0.12$ and $p>0.25$ for *per1b* and *arntl1a*, respectively, by Tukey's test). The LD data points are significantly different from each other ($p<1x10\text{-}6$ for *per1b* and *arntl1a* by Tukey's test). 24 larvae were pooled for each sample and the mean ± SEM of triplicate samples at each time point is shown. (I-Q). Larvae raised in LD (lights on at 9 am and off at 11 pm) or DD were fixed at the indicated time points beginning at 6 pm at 6 dpf. Dissected brains were processed for fluorescent *in situ* hybridization using a probe specific for *per1b* and were counterstained with DAPI to label nuclei. In LD, *per1b* expression oscillates with a peak at 12 pm and trough at 12 am (I, K, M, O). In DD, *per1b* expression levels are similar at all time points (J, L, N, P). White boxes in I and I' indicate region of the hypothalamus that is shown at higher magnification in (I"-P") and (I"'-P"'). Yellow ovals in I" and I"' indicate region used to quantify *per1b* fluorescence in all samples. Representative images are shown. Scale bar=50 μm. (Q). Quantification of total *per1b* fluorescence intensity in a 23 μm thick confocal image stack. *per1b* expression oscillates in LD (peak:trough ratio=10.7, $p<0.01$ by Tukey's test) but not in DD (peak:trough ratio=1.2, $p=0.77$ by Tukey's test). Mean ± SEM is shown. Three brains were quantified for each lighting condition at each time point.

loss of melatonin. We tested this using two approaches. We first asked whether melatonin is required for behavioral circadian rhythms using the videotracker assay. To monitor circadian rhythms in "free-running" conditions, we entrained larvae in LD and then shifted them to DD. In these conditions, WT larvae maintain molecular and behavioral rhythms (Figure 2.9A, B, K, L) (Kaneko and Cahill, 2005). While *aanat2*$^{-/-}$ larvae had smaller circadian amplitude due to increased locomotor activity during subjective night (Figures 6A,B and 8A, H), the period length and phase were similar to controls (Figure 2.7C, D). Second, we used transgenic fish in which the period 3 promoter regulates expression of luciferase (*per3*:luc), an in vivo reporter of molecular rhythms in intact larvae (Kaneko and Cahill, 2005). We found that *per3*:luc amplitude, period length and phase were not significantly different for any genotype (Figure 2.7E-H). To confirm this result, we isolated RNA from WT and *aanat2*$^{-/-}$ larvae and performed RT-qPCR for the circadian genes per1b and arntl1a. Circadian oscillation of both genes was indistinguishable between WT and *aanat2*$^{-/-}$ (Figure 2.9K,L), indicating that *aanat2*$^{-/-}$ larvae have normal molecular rhythms. Together the observation that *aanat2*$^{-/-}$ larvae exhibit normal locomotor activity period length and phase in LD (Figure 2.1A), this indicates that endogenous melatonin is not required to initiate or maintain circadian rhythms. We conclude that melatonin functions downstream of the circadian clock to promote sleep in zebrafish.

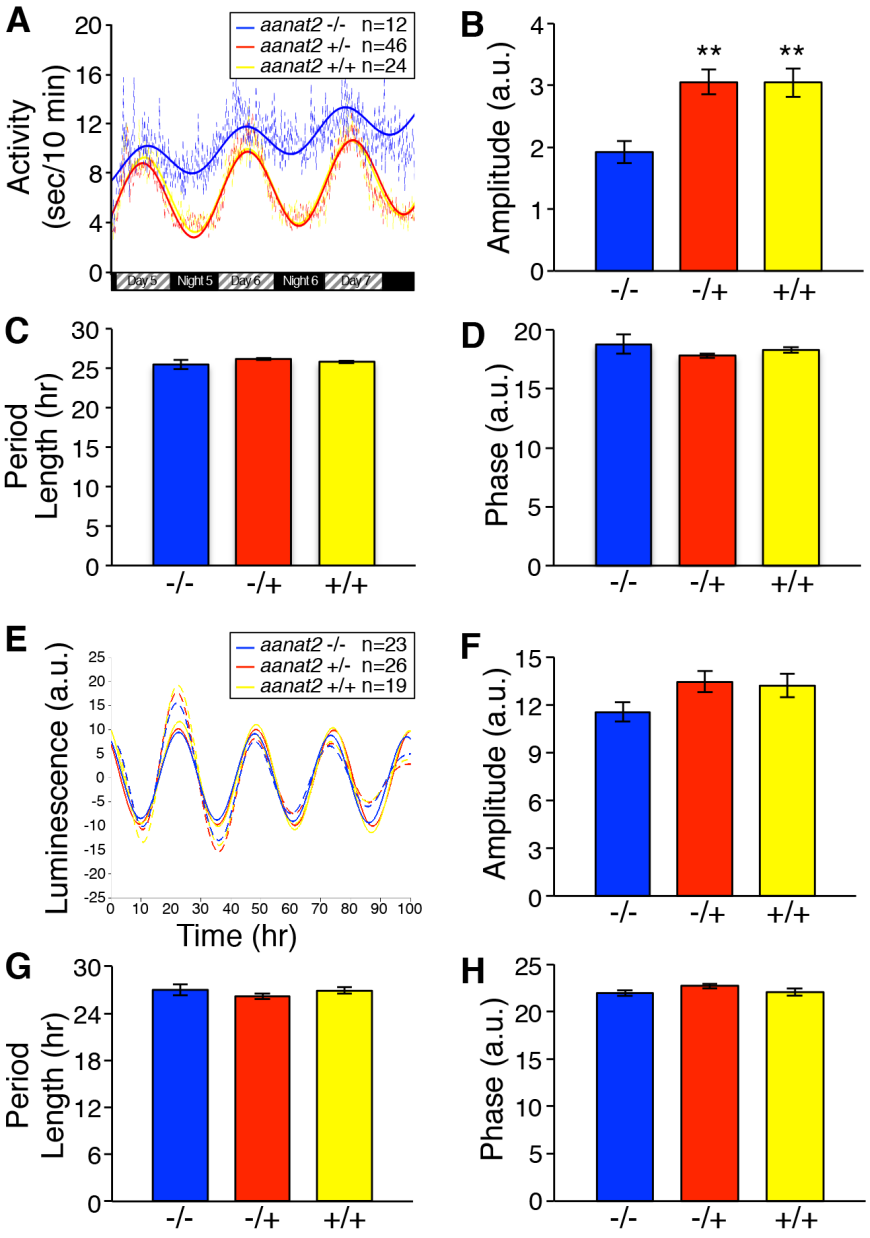

Figure 2.7. aanat2 is not required for behavioral or molecular circadian rhythms. (A) Larvae raised in LD and monitored in DD maintain locomotor activity circadian rhythms. Locomotor activity data (dashed lines) was fit to damped cosine curves (solid lines) to quantify circadian amplitude (B), period length (C) and phase (D). *aanat2-/-* larvae have reduced amplitude due to increased locomotor activity during subjective night, but period length and phase are indistinguishable from sibling controls. Black and hatched boxes indicate subjective night and day, respectively. (E-H) per3-luc larvae were raised in LD and luminescence recorded during 100 hours in DD. (E) Dashed and solid lines indicate luminescence data and damped cosine curve fits, respectively. (F-H) Circadian rhythm amplitude, period length and phase are not significantly different for any genotype. Bar graphs represent mean ± SEM. a.u., arbitrary units. **, $p<0.01$ compared to *aanat2-/-* by Dunnett's test. See also Figure 2.8.

Melatonin is required for the circadian regulation of sleep

Our results suggest that melatonin may mediate process C, which determines when sleep occurs during the circadian cycle (Borbély, 1982). To address this possibility, we entrained larvae in LD and transferred them to DD to remove masking effects of light on behavior. Under these conditions, *aanat2*$^{-/-}$ larvae, but not sibling controls, exhibited similar amounts of sleep during subjective day and night (Figure 2.8A-C), despite the maintenance of normal molecular rhythms (Figures 2.7E-F, 2.9K, L). This indicates that melatonin is required for circadian regulation of sleep. Combined with the observation that exogenous melatonin is sufficient to induce daytime sleep (Figure 2.1H,I) (Zhdanova et al., 2001), this suggests that melatonin mediates process C in zebrafish larvae.

Adenosine may act downstream of Melatonin to promote sleep at night

Exogenous melatonin can induce adenosine production in the mammalian forebrain (Zamorskii and Pishak, 2003). To test whether adenosine mediates sleep promotion by melatonin, we treated *aanat2*$^{-/-}$ larvae and their *aanat2*$^{+/-}$ siblings with the adenosine receptor agonist 5'-N- ethylcarboxamido-adenosine (NECA). NECA treatment decreased locomotor activity in *aanat2*$^{+/-}$ larvae during the day (Figures 2.8D and 2.10A) but had no effect on activity or sleep at night (Figure 2.8D-2.8G; arrows indicate transient artifacts caused by pipetting compounds into the plate), presumably because adenosine receptors are maximally activated at night. In contrast, NECA increased sleep and decreased activity at night for *aanat2*$^{-/-}$ larvae similarly to their *aanat2*$^{+/-}$ siblings, (Figure 2.8F,G), thus rescuing the mutant phenotype. As an alternative approach to modulate adenosine signaling, we treated larvae with ABT702, a small molecule inhibitor of

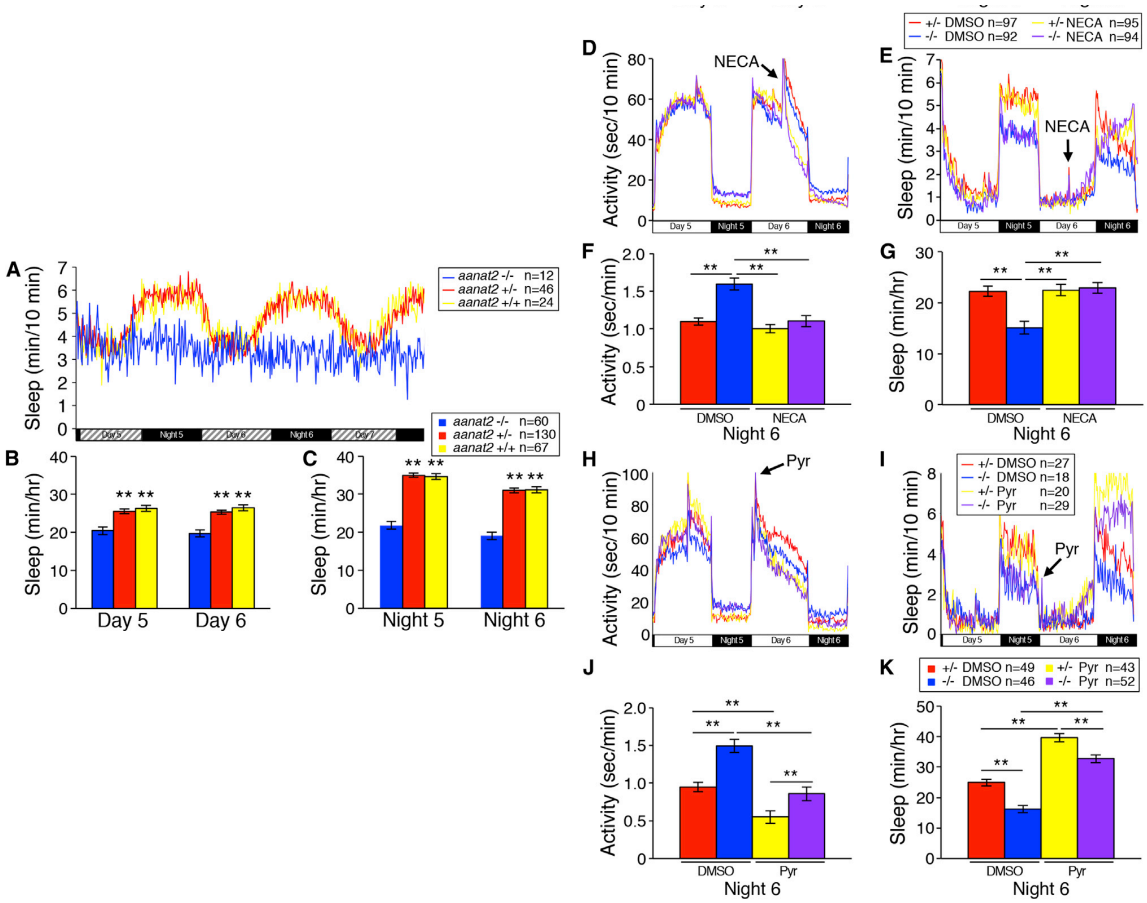

Figure 2.8. Melatonin is required for circadian regulation of sleep and may promote sleep via adenosine signaling. (A-C) Larvae were raised in LD and tested in DD. aanat2-/- larvae, but not sibling controls, lack circadian sleep oscillations. Black and hatched boxes represent subjective night and day, respectively. There is no significant difference in amount of sleep for aanat2-/-larvae during each subjective day (B) and night (C) ($p>0.22$ by Tukey's test). (D-G) Larvae were treated with vehicle or 50 μM NECA on day 6. On night 6, vehicle-treated aanat2-/- larvae are more active (D,F) and sleep less (E,G) than vehicle-treated aanat2+/- larvae. In contrast, NECA- treated *aanat2-/-* larvae exhibit the same amount of activity (D,F) and sleep (E,G) as NECA- treated *aanat2+/-* larvae. (H-K) Larvae were treated with vehicle control or 10 μM pyrilamine on day 6. Pyrilamine decreases locomotor activity (H,J) and increases sleep (I,K) for *aanat2-/-* and *aanat2+/-* larvae to a similar extent. Bar graphs represent mean ± SEM. **, $p<0.01$ compared to *aanat2-/-* (B,C), *aanat2-/-* DMSO (F,G), or for the indicated comparisons (J,K) by Dunnett's (B,C,F,G) or Tukey's test (J,K). Arrows indicate transient artifacts due to pipetting of compounds. See also Figure 8.

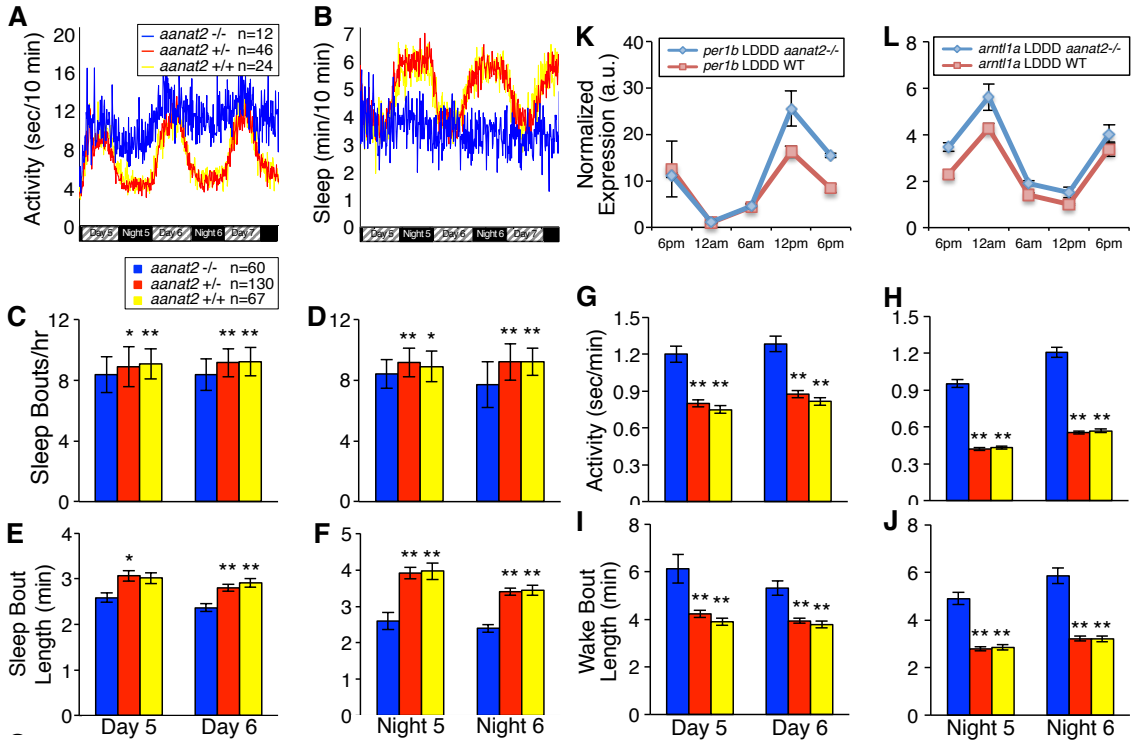

Figure 2.9. Melatonin is required for the circadian regulation of sleep (Related to Figure 2.8) (A-J) Graphs are based on data shown in Figures 7A-7C. (A, B) WT and heterozygous mutants raised in LD and tested in DD maintain locomotor activity and sleep circadian rhythms, but *aanat2-/-* larvae lack sleep circadian oscillations. Black and hatched boxes indicate subjective night and day, respectively. *aanat2-/-* larvae exhibit slightly fewer sleep bouts (D), shorter sleep bouts (F), more locomotor activity (H) and longer wake bouts (J) during subjective night. Similar but generally weaker phenotypes are observed during subjective day (C, E, G, I). Data are from one representative experiment (A, B) or combined from three (C-J) experiments. Bar graphs represent median ± MAD (C, D) and mean ± SEM (E-J). *, $p<0.05$; **, $p<0.01$ compared to *aanat2-/-* by Steel-Dwass (C, D) or Dunnett's test (E-J). (K, L) *aanat2-/-* and WT larvae raised in LD maintain circadian rhythms in DD (LDDD). WT (red) and *aanat2-/-* (blue) larvae were raised in LD and shifted to DD at 11 pm on the fourth night of development. Samples were collected at 6-hour intervals from 6 pm on day 5 until 6 pm on day 6. The expression of *per1b* (K) and *arntl1a* (L) oscillates for both WT and *aanat2-/-* larvae (peak:trough ratio is 16 for WT and 22 for *aanat2-/-* for *per1b*, and 4.3 for WT and 3.7 for *aanat2-/-* for *arntl1a*). 24 larvae were pooled for each sample and the mean ± SEM of triplicate samples at each time point is shown.

adenosine kinase that increases extracellular adenosine levels, and thus adenosine signaling (Brown et al., 2012). Similar to NECA, ABT702 (Figure 2.10B) had no effect on locomotor activity or sleep of *aanat2*$^{+/-}$ larvae at night, but rescued the activity and sleep phenotypes of *aanat2*$^{-/-}$ larvae (Figure 2.10D-F). These results suggest that melatonin may promote sleep, at least in part, by inducing adenosine signaling. To test whether these drugs failed to increase sleep at night in *aanat2*$^{+/-}$ larvae because they were already sleeping maximally, we promoted sleep via a different mechanism. Histamine is an arousing neuromodulator via mechanisms that are not well understood (Panula and Nuutinen, 2013). Inhibition of the histamine 1 receptor (H1R) is sedating in mammals (Panula and Nuutinen, 2013) and zebrafish (Renier et al., 2007). In contrast to NECA and ABT702, the H1R antagonist pyrilamine increased sleep and decreased activity in both *aanat2*$^{-/-}$ and *aanat2*$^{+/-}$ larvae to a similar extent (Figures 2.8H-K and 2.10C). This indicates that the failure of NECA to increase nighttime sleep in *aanat2*$^{+/-}$ larvae was not because the larvae were already sleeping maximally, and suggests that histamine acts parallel to, rather than downstream of, melatonin in regulating sleep. Together, our results are consistent with a model in which adenosine acts downstream of melatonin to promote sleep at night, thus potentially linking homeostatic and circadian regulation of sleep (Figure 2.11).

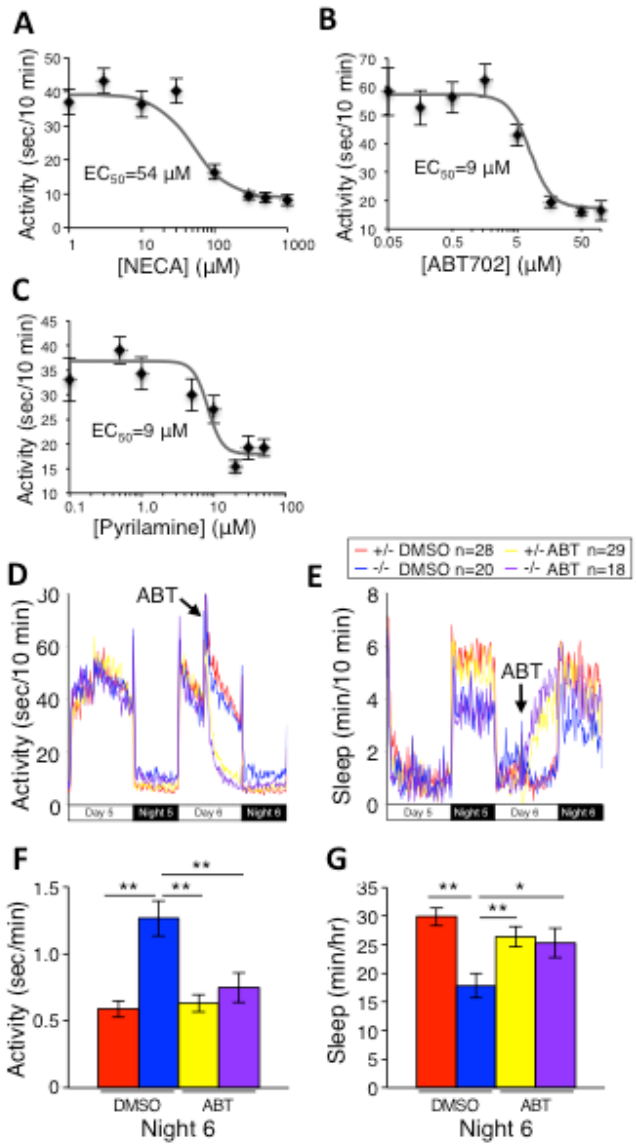

Figure 2.10. Drug dosage curves and melatonin may act through adenosine for its effects on sleep (Related to Figure 2.8) (A-C) Dose response curves for NECA (A), ABT702 (B) and pyrilamine (C). Each data point represents the average locomotor activity of 8 larvae during the day following addition of each drug. Drugs were used at or above the EC50 concentration for behavioral experiments. (D-G) Larvae were treated with either vehicle or 30 µM ABT702 starting on day 6. On night 6, vehicle-treated *aanat2*-/- larvae are more active (D, F) and sleep less (E, G) than vehicle-treated *aanat2*+/- larvae. In contrast, ABT702-treated *aanat2*-/- larvae exhibit the same amount of activity (D, F) and sleep (E, G) as ABT702-treated *aanat2*+/- larvae. Bar graphs represent mean ± SEM. *, $p<0.05$; **, $p<0.01$ compared to *aanat2*-/- DMSO by Dunnett's test. Note that there are no significant differences in locomotor activity or sleep on night 6 between ABT702-treated *aanat2*+/- and *aanat2*-/- larvae, but there are significant differences between DMSO- and ABT702-treated *aanat2*-/- larvae. Also note that the transient apparent increase in locomotor activity observed upon adding the drug or vehicle is an artifact due to compounds being pipetted into the plate.

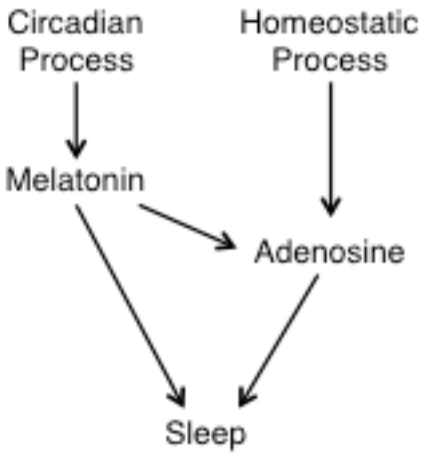

Figure 2.11. Melatonin is required for the circadian regulation of sleep and may act via adenosine (model) (Related to Figure 2.8). Model for circadian regulation of sleep by melatonin. The circadian clock regulates expression of *aanat2*, and thus melatonin production, in the pineal gland, with high levels at night and low levels during the day (Fisher et al., 2013). The high level of melatonin at night promotes the initiation and maintenance of sleep. Extracellular adenosine accumulates in specific brain regions in proportion to time spent awake, and is thought to play a role in the homeostatic regulation of sleep (Brown et al., 2012). Melatonin may promote sleep, at least in part, by promoting adenosine signaling, thus linking homeostatic and circadian regulation of sleep. Melatonin likely also promotes sleep via additional mechanisms because extracellular adenosine levels decline during sleep (Brown et al., 2012) whereas the *aanat2* mutant phenotype persists throughout the night. Melatonin-induced adenosine signaling may predominantly occur at the beginning of the night, when melatonin levels are still rising, to promote a rapid and robust wake to sleep transition. We found no evidence that melatonin feeds back to the circadian clock to regulate circadian rhythms.

Discussion

Melatonin is widely used as a sleep aid and has been cited as a regulator of circadian rhythms (Elbaz et al., 2013). However, despite decades of study, the role of melatonin in regulating sleep and circadian rhythms is controversial (Fisher et al., 2013) and the function of endogenous melatonin is unknown. Here we describe the first diurnal vertebrate genetic loss of function model for melatonin. We show that *aanat2*$^{-/-}$ zebrafish larvae take twice as long to fall asleep and sleep only half as much as controls at night in LD conditions. This effect is surprisingly large since exogenous melatonin has relatively subtle sleep promoting effects in humans compared to prescribed hypnotics (Brzezinski et al., 2005; Buscemi et al., 2006), which has led some to argue that melatonin is not an important sleep regulator (van den Heuvel et al., 2005). However, most hypnotics inhibit neuronal activity throughout the brain by activating GABAA receptors, which is not a physiologically relevant mechanism of sleep promotion (Zhdanova, 2005). Indeed, such an overpowering mechanism of sleep induction would be maladaptive. Further, while exogenous melatonin may be a relatively weak sedative, it does not necessarily follow that endogenous melatonin does not play an important role in sleep. Comparing the importance of endogenous melatonin in humans and zebrafish will require more potent and specific melatonin receptor antagonists. While melatonin may play a more important role in promoting sleep in zebrafish, our results demonstrate that endogenous melatonin plays a significant role in promoting initiation and maintenance of sleep at night in a diurnal vertebrate.

It has been proposed that melatonin promotes sleep indirectly by phase-advancing the circadian clock (Arendt, 2003) or inhibiting the circadian drive for wakefulness

(Scheer and Czeisler, 2005). If these hypotheses are correct, *aanat2*$^{-/-}$ larvae should have little or no sleep phenotype in the absence of entrained circadian rhythms. We tested this hypothesis by raising larvae in constant darkness, which abolished cellular circadian oscillations, at least in the brain. The *aanat2*$^{-/-}$ sleep phenotype persisted under these conditions, suggesting that endogenous melatonin does not promote sleep by modulating the circadian clock, but rather directly affects the sleep regulatory system. Indeed, while exogenous melatonin can entrain the circadian clock in free- running animals (Lockley et al., 2000; Sack et al., 2000) and phase-shift the clock in some contexts (Lewy et al., 1992), we found that endogenous melatonin is not required to initiate or maintain molecular or behavioral circadian rhythms in zebrafish. This observation does not support the hypothesis, based on exogenous melatonin, that endogenous melatonin regulates circadian rhythms (Elbaz et al., 2013). Rather, our data suggest that melatonin acts downstream of the clock to promote sleep.

A prominent model proposes that sleep is regulated by a homeostatic process responding to internal cues for sleep need (process S), and a circadian process responding to external cues (process C) (Borbély, 1982). Evidence in mammals suggests that factors such as adenosine, nitric oxide and prostaglandin D2 play important roles in mediating the homeostatic process (reviewed in Brown et al., 2012). However, while the circadian clock mechanism has been described in detail (Fisher et al., 2013), molecules that convey circadian information to regulate sleep are largely unknown. A factor mediating process C should fulfill three criteria. First, the clock should regulate the level or activity of the factor. Second, administration of the factor should induce sleep during the circadian waking period, but not the sleep period. Third, loss of the factor should abolish circadian

regulation of sleep. Peptides whose expression oscillates in a circadian manner, and whose overexpression inhibits activity or promotes sleep during the circadian waking period, have been identified in nocturnal rodents, including cardiotrophin-like cytokine (Kraves and Weitz, 2006), transforming growth factor alpha (Kramer et al., 2001) and prokineticin 2 (Cheng et al., 2002). However, loss-of-function studies have revealed little or no effect on the circadian regulation of activity or sleep (Hu et al., 2007; Kraves and Weitz, 2006; Li et al., 2006; Roberts et al., 2006). Melatonin is an alternative candidate for mediating process C since, similar to these peptides, the circadian clock regulates its production (Klein, 2007) and it can induce sleep in some contexts (Fisher et al., 2013). Indeed, exogenous melatonin potently increases sleep and decreases locomotor activity in zebrafish larvae during the day (Figures 2.1G- L) (Zhdanova et al., 2001), and circadian regulation of sleep is abolished in aanat2$^{-/-}$ larvae. These results suggest that melatonin mediates process C in the diurnal zebrafish animal model. This discovery may have important implications for the treatment of sleep and circadian rhythm disorders in humans.

Because melatonin is produced at night in diurnal and nocturnal animals, and administration of physiological levels of melatonin does not promote sleep in nocturnal animals (Fisher et al., 2013), nocturnal and diurnal animals likely use different mechanisms for circadian regulation of sleep (Zhdanova, 2005). This idea is supported by the observation that most nocturnal mouse strains used for genetic studies produce little or no melatonin, (Goto et al., 1989) yet have circadian control of sleep. Mutation of *aanat* in melatonin-proficient nocturnal and diurnal mammals will clarify melatonin's role in regulating mammalian sleep.

An open question raised by the two-process model (Borbely, 1982) is how homeostatic and circadian cues are integrated. Similar to melatonin, we found that activating adenosine signaling promotes sleep and inhibits activity during the day, but has no effect on sleep at night in WT (data not shown) and *aanat2*$^{+/-}$ larvae. In contrast, activating adenosine signaling increases sleep and decreases activity at night in *aanat2*$^{-/-}$ larvae to the same level as their *aanat2*$^{+/-}$ siblings. This result is unlikely due to a ceiling effect for sleep or parallel modulation of sleep by melatonin and adenosine because increasing nighttime sleep using a different approach, using a histamine H1R antagonist, increases nighttime sleep for both *aanat2*$^{+/-}$ and *aanat2*$^{-/-}$ larvae to a similar extent. These results suggest the sleep-promoting effect of endogenous melatonin may be mediated, at least in part, by adenosine signaling, and suggest a potential mechanism linking homeostatic and circadian regulation of sleep. This hypothesis must be further tested using genetics and measurements of adenosine levels, which will be challenging in the zebrafish due to its large number of adenosine receptor genes and its small brain size. Application of genome editing technologies to diurnal melatonin-proficient mammals would allow the use of genetics and measurement of adenosine levels using available technologies (Porkka-Heiskanen et al., 1997; Schmitt et al., 2012).

Finally, we note that reduced melatonin levels are associated with aging, mood disorders and autism (Hardeland, 2012). An improved understanding of how endogenous melatonin interacts with the sleep system and other aspects of physiology from the aanat2 mutant may lead to novel therapies for these disorders.

Materials and methods

Zebrafish Genetics

aanat2 mutant zebrafish were generated using the TAL effector nuclease (TALEN) method as described (Reyon et al., 2012) using plasmids obtained from Addgene. The TALEN target sites were 5'-TGGTGGCCTTCATCATT-3' and 5'-TGTTCTAGTTTCTCTT-3'. Two mutants were isolated and tested. Mutant d10 contains a 10 bp deletion (nucleotides 295-304 of the open reading frame: 5'-TCTGGCTGGG-3'). Mutant d11 contains an 11 bp deletion (nucleotides 296- 306 of the open reading frame: 5'-CTGGCTGGGAT-3'). The mutations result in a change in reading frame after amino acid 98 and a premature stop codon after amino acid 102 (d10) or 98 (d11), compared to 210 amino acids for the WT protein. The mutants exhibited identical phenotypes and mutant d10 was used for all reported experiments. aanat2 mutants were genotyped using the primers 5'-CAGTGTCCTGAGCTGTCCAT-3' and 5'-CGAAAGGCACATCACCATC-3'. Mutant and WT PCR products (162 bp and 172 bp, respectively) were distinguished by running the PCR reaction on a 4% agarose gel. aanat1 mutants were generated using the Cas9/CRISPR method (Hwang et al., 2013) using plasmids obtained from Addgene and the sgRNA 5'-GGAGCGTGTGTCGGCGCTGG-3'. The aanat1 mutant contains a 40 bp deletion (nucleotides 67-106 of the open reading frame: 5'-CGCCAGCGCCGACACACGCTCCCAGCAAGCGAGTTTCGCT-3'). This deletion results in a change in reading frame after amino acid 23 and a premature stop codon after amino acid 78, compared to 204 amino acids for the WT protein. aanat1 mutants were genotyped using the primers 5'-GTAGTGAGCGCACTGCCTTT-3' and 5'-

CACACATATGACCACACAAACCT- 3'. Mutant and WT PCR products (168 bp and 208 bp, respectively) were distinguished by running the PCR reaction on a 3% agarose gel. For both aanat1 and aanat2 mutants, the frame shifts and premature stop codons are 5' to domains critical for enzyme activity in the sheep aanat ortholog (Hickman et al., 1999; Scheibner et al., 2002), indicating that the mutant proteins are likely to be non-functional. The Tg(aanat2:CFP-NTR) transgenic line was generated by subcloning the 5' and 3' aanat2 enhancer elements (Gothilf et al., 2002) 5' and 3' to a CFP-NTR transgene (Curado et al., 2007) in a vector containing Tol2 transposase arms. The plasmid was coinjected with Tol2 transposase mRNA and transgenic lines were identified based on CFP fluorescence.

Behavioral Analysis

Larval zebrafish were raised on a 14:10 hour light:dark cycle at 28.5OC with lights on at 9 am and off at 11 pm. Individual larvae were placed into each well of a 96-well plate (7701-1651, Whatman) containing 650 μL of E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4) (Prober et al., 2006). In most experiments, plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. The sealing process introduces air bubbles in some wells, which are discarded from analysis. Locomotor activity was monitored using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch Monochrome camera (Dragonfly 2, Point Grey) fitted with a variable- focus megapixel lens (M5018-MP, Computar) and infrared filter. The movement of each larva was recorded using the quantization mode. The 96-well plate and camera were housed inside

a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and illuminated with white lights from 9 am to 11 pm. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5OC. The parameters used for detection were: detection threshold, 15; burst, 25; freeze, 3; bin size, 60 seconds. Larvae were exposed to 10 μM melatonin (3550, Tocris Bioscience) or 10 μM pyrilamine (P5514, Sigma Aldrich) by adding 100 μL of a 65 μM stock solution (in 0.02% and 0.065% DMSO for melatonin and pyrilamine, respectively) to E3 embryo medium during the experiment. Larvae were exposed to 50 μM NECA (E2387, Sigma Aldrich) by adding 100 μL of a 325 μM stock solution containing 1% DMSO to E3 embryo medium. Larvae were exposed to 30 μM ABT 702 dihydrochloride (2372, Tocris) by adding 100 μL of a 195 μM stock solution containing 0.65% DMSO to E3 embryo medium. The same concentration of DMSO was used as vehicle control for each drug experiment. For dose-response experiments, curve fitting was performed using a 4 parameter logistic nonlinear curve model and EC50 values were calculated using JMP 10.0 (SAS Institute Inc.).

Arousal Threshold Assay

The videotracking system was modified with an Arduino (http://www.arduino.cc/) based automated driver to control a solenoid (28P-I-12, Guardian Electric) delivering a tap to a 96-well plate containing larvae. Taps were applied from 12:30 am to 7:30 am the 5th night of development with an inter-trial-interval of 1 minute to measure overall arousal threshold or 5 minutes to assay arousal during sleep. Previous studies showed that a 30 second interval between repetitive stimuli is sufficient to prevent behavioral habituation

(Woods et al., 2014). In experiments where stimulus intensities were varied, 14 different intensities were applied in a random manner, with 30 trials at each intensity. For experiments where the stimulus was applied every 5 minutes, 60 trials were performed. The response of larvae to the stimuli was monitored using the videotracking software and subsequently analyzed in Matlab (version R2013a, TheMathworks, Inc) and Excel (Microsoft). Statistical analysis was performed using the Variable Slope log(dose) response curve fitting module of Prism (Graphpad).

period3-luciferase Assay

This assay was performed as described (Kaneko and Cahill, 2005), with some modifications. Larval zebrafish harboring a per3-luc reporter (Kaneko and Cahill, 2005) were raised on a 14:10 hour light:dark cycle at 22°C with lights on at 9 am and off at 11 pm. At 6 days post-fertilization (dpf), individual larvae were placed in each well of 96-well plates (T-2996-075, Greiner) containing 50 μL Holtfreter's solution (59 mM NaCl, 0.67 mM KCl, 0.76 mM CaCl2, and 2.4 mM NaHCO3, pH 7.0), 0.5 mM D-luciferin (L8220, Biosynth Chemistry and Biology) and 0.013% Amquel Instant Water Detoxifier (Kordon), and sealed with an optical adhesive film (4311971, Applied Biosystems). Bioluminescence in each well was recorded for 3 seconds every hour for 100 hours in constant darkness using a plate reader (M1000 Pro, Tecan).

Data analysis

Videotracker sleep/wake data was processed using custom PERL and Matlab (version R2013a, The Mathworks, Inc) scripts. Any one minute period with no movement was

defined as one minute of sleep (Prober et al., 2006). A sleep bout was defined as a continuous string of sleep minutes. Sleep latency was defined as the length of time from lights on or off to the start of the first sleep bout. Average activity was defined as the average amount of detected activity in seconds/minute, including all rest bouts. These parameters were calculated for each experimental day and night. Luminescence and behavioral data was processed for circadian analysis using custom Matlab scripts. Raw data were fit to a damped cosine curve as described (Hirota et al., 2008). Statistical tests were performed using JMP 10.0 (SAS Institute Inc.).

Pineal Ablation

Cell ablation using nitroreductase (NTR) was performed as described (Curado et al., 2007) with some modifications. aanat2-CFP-NTR lines containing multiple copies of the transgene and exhibiting high levels of CFP fluorescence in the pineal gland, with no non-pineal expression, were selected and incrossed. Embryos were raised in E3 medium until 60 hours post-fertilization (hpf), at which point they were separated into pools of larvae that exhibited strong or no CFP expression. Each pool was treated with 15 mM metronidazole (MTZ, 46461, Sigma Aldrich) in 0.2% DMSO/E3 medium for 20 hours (60-80 hpf). Larvae were then thoroughly rinsed with fresh E3 medium and maintained in E3 medium for 24 hours, followed by a second treatment with 15 mM MTZ for 20 hours (108-128 hpf). Larvae were then transferred to fresh E3 medium and screened for efficacy of pineal ablation using a fluorescence stereomicroscope (m205c, Leica Microsystems Inc.). Larvae with the weakest CFP fluorescence, as well as CFP negative siblings that were similarly treated and screened, were selected for behavioral monitoring

that was initiated at 134 hpf. To quantify the extent of pineal ablation, aanat2-CFP-NTR animals treated with MTZ or DMSO vehicle control as described above were anesthetized (0.016% w/v tricaine methane sulfonate, A5040, Sigma Aldrich) and mounted in 0.8% low melting agarose (16520-050, Invitrogen), and imaged on a confocal microscope (Zeiss 780 LSM, 20x dipping objective with 3x digital zoom). Imaging was performed between 11 pm and 7 am. The number of CFP-expressing cells was counted using the contrast between the nucleus and cytoplasm to distinguish individual cells for 3 animals at each time point. Double-fluorescent in situ hybridizations were performed using digoxygenin (DIG)- and 2,4-dinitrophenol (DNP)-labeled riboprobes and the TSA Plus DNP System (NEL741001, PerkinElmer). Samples were mounted in 50% glycerol/PBS and imaged using a confocal microscope (Zeiss 780 LSM).

Melatonin ELISA

The melatonin ELISA was performed as described (de Borsetti et al., 2011; Kazimi and Cahill, 1999). Triplicate samples containing 5 larvae each were homogenized in 0.5 mL 0.1 N NaOH at 6 am on night 5 and 6 pm on day 6. One mL methylene chloride (9315, J.T. Baker) was added to each sample, followed by vortexing for 30 seconds and centrifugation at 4250 g for 3 hours at 4°C. The organic phase was collected and 0.5 mL 0.1 N NaOH was added, followed by vortexing for 30 seconds and centrifugation for 20 minutes at 4250 g at 4°C. The organic phase was collected and 0.15 g sodium sulfate (SX0760, EMD Millipore) was added to each sample, followed by another 30 second vortex and 20 minute centrifugation at 4250 g at 4°C. One mL of the organic phase from each sample was transferred to a 15 mL falcon tube and wrapped in foil to protect from

light. The solution in each tube was evaporated using a rotary evaporator with a room temperature water bath. The residue was dissolved in 200 μL phosphate buffered saline (PBS) with 0.1% gelatin (214340, Becton Dickinson). Melatonin levels were assayed using a melatonin saliva ELISA kit (101720-920, Alpco Diagnostics) according to the manufacturer's instructions.

Pineal Serotonin (5-HT) Quantification

Larvae were raised in E3 medium until 6 dpf and fixed at 6 pm (day) or 6 am (night) in 4% paraformaldehyde (PFA) overnight at 4oC. Samples were washed in 0.25% Triton X-100 in PBS (PBSTx). Brains were dissected while leaving skin on the dorsal side of the brain intact to avoid removing pineal gland cells, and treated with 1 mg/ml collagenase for 45 minutes at room temperature. Samples were blocked in 2% normal goat serum/2% DMSO in PBSTx for at least two hours at room temperature. Antibody incubations were performed in blocking solution overnight at 4oC. Rabbit anti-serotonin (1:1000, S5545; Sigma) and Alexa-488 anti-rabbit (1:500, A11034, Life Technologies) antibodies were used. Samples were washed in PBSTx and mounted in 50% glycerol/PBS. Pineal imaging was performed using a confocal microscope (Zeiss 780 LSM, 40x objective, 2x digital zoom) using the same settings for all samples. ImageJ was used to create a summation z-projection for each confocal stack and the integrated pixel density for the entire pineal gland was used to quantify 5-HT fluorescence.

per1b Fluorescent In Situ Hybridization

Larvae were raised in E3 medium in either LD (lights on at 9 am and off at 11 pm) or DD (starting at 6 hpf) until 6 dpf. Samples were fixed in 4% PFA overnight at room temperature and fluorescent in situ hybridization was performed on dissected brains using a DIG-labeled antisense riboprobe specific for per1b (Dekens and Whitmore, 2008) and the TSA Plus System (NEL741001, PerkinElmer). Samples were developed using Cy3 amplification reagent at 1:200 for 10 minutes and stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (268298, EMD Millipore) in 2% DMSO PBSTx for at least 4 hours at room temperature. Samples were then washed in PBSTx and mounted in 50% glycerol/PBS. Imaging was performed using a confocal microscope using the same settings for all samples (Zeiss 780 LSM, 10x and 40x objectives). 31 optical sections of 0.75 μm thickness each (23.25 μm total) were acquired for each sample. ImageJ was used to create a summation z-projection for each stack and the integrated pixel density within a region of interest of the same size for all samples was used to quantify total Cy3 fluorescence.

Quantitative PCR

Larval zebrafish were raised on either a 14:10 hour light:dark cycle at 28.5°C with lights on at 9 am and off at 11 pm, or in constant darkness. At 4 dpf, individual larvae were placed into each well of 96-well plates (7701-1651, Whatman) in dim red lighting and placed into constant darkness. Total RNA was collected using Trizol reagent (15596-026, Life Technologies) from 24 pooled larvae starting at 6 pm on day 5 every 6 hours for 24 hours. cDNA was synthesized from 5 μg of total RNA using Superscript III Reverse

Transcriptase (18080-051, Invitrogen) and quantitative PCR was carried out using SYBR green master mix (4364346, Life Technologies) in an ABI PRISM 7900HT (Life Technologies) instrument. Δ Ct was calculated using ribosomal protein l13α (rpl13α) as a reference gene. Relative expression levels were plotted by determining ΔΔCt by normalizing to the LD sample or the WT LDDD sample (for Figures S3G-S3H and S4K-S4L, respectively) with the highest ΔCt value for each gene.

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