Neuron

Supplemental Information

Melatonin Is Required for the Circadian Regulation of Sleep

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Figure S1

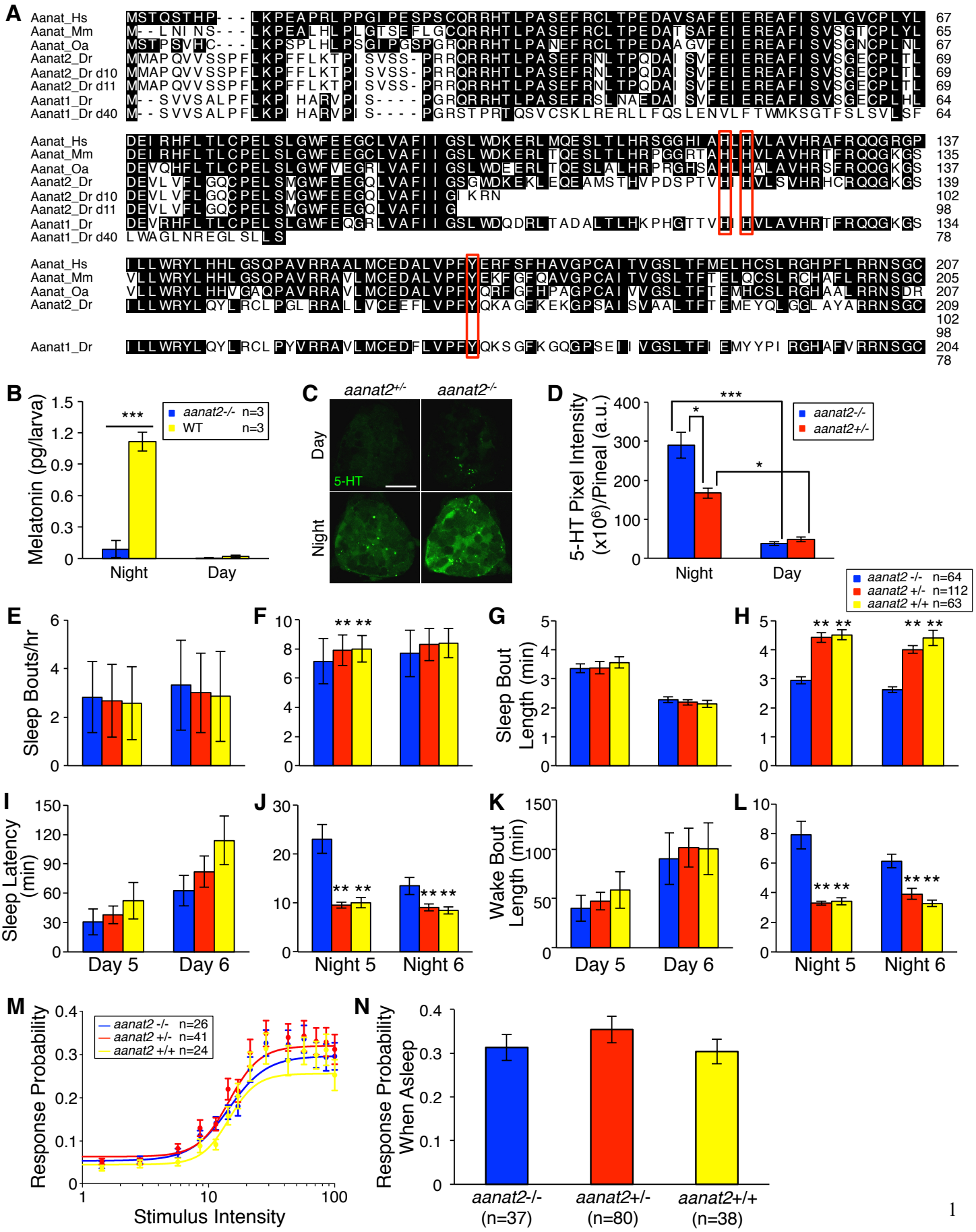

A
Aanat_Hs
Aanat_Mm
Aanat_Oa
Aanat2_Dr
Aanat2_Dr d10
Aanat2_Dr d11
Aanat1_Dr
Aanat1_Dr d40
B
Melatonin (pg/larva)
aanat2-/- n=3
WT n=3
Night
Day
C
aanat2+/-
aanat2-/-
Day
Night
5-HT
D
5-HT Pixel Intensity (x10^6)/Pineal (a.u.)
aanat2-/-
aanat2+/-
Night
Day
aanat2 -/- n=64
aanat2 +/- n=112
aanat2 +/+ n=63
E
Sleep Bouts/hr
F
G
Sleep Bout Length (min)
H
I
Sleep Latency (min)
Day 5
Day 6
J
Night 5
Night 6
K
Wake Bout Length (min)
L
M
Response Probability
aanat2 -/- n=26
aanat2 +/- n=41
aanat2 +/+ n=24
Stimulus Intensity
N
Response Probability When Asleep
aanat2-/- (n=37)
aanat2+/- (n=80)
aanat2+/+ (n=38)

Figure S2

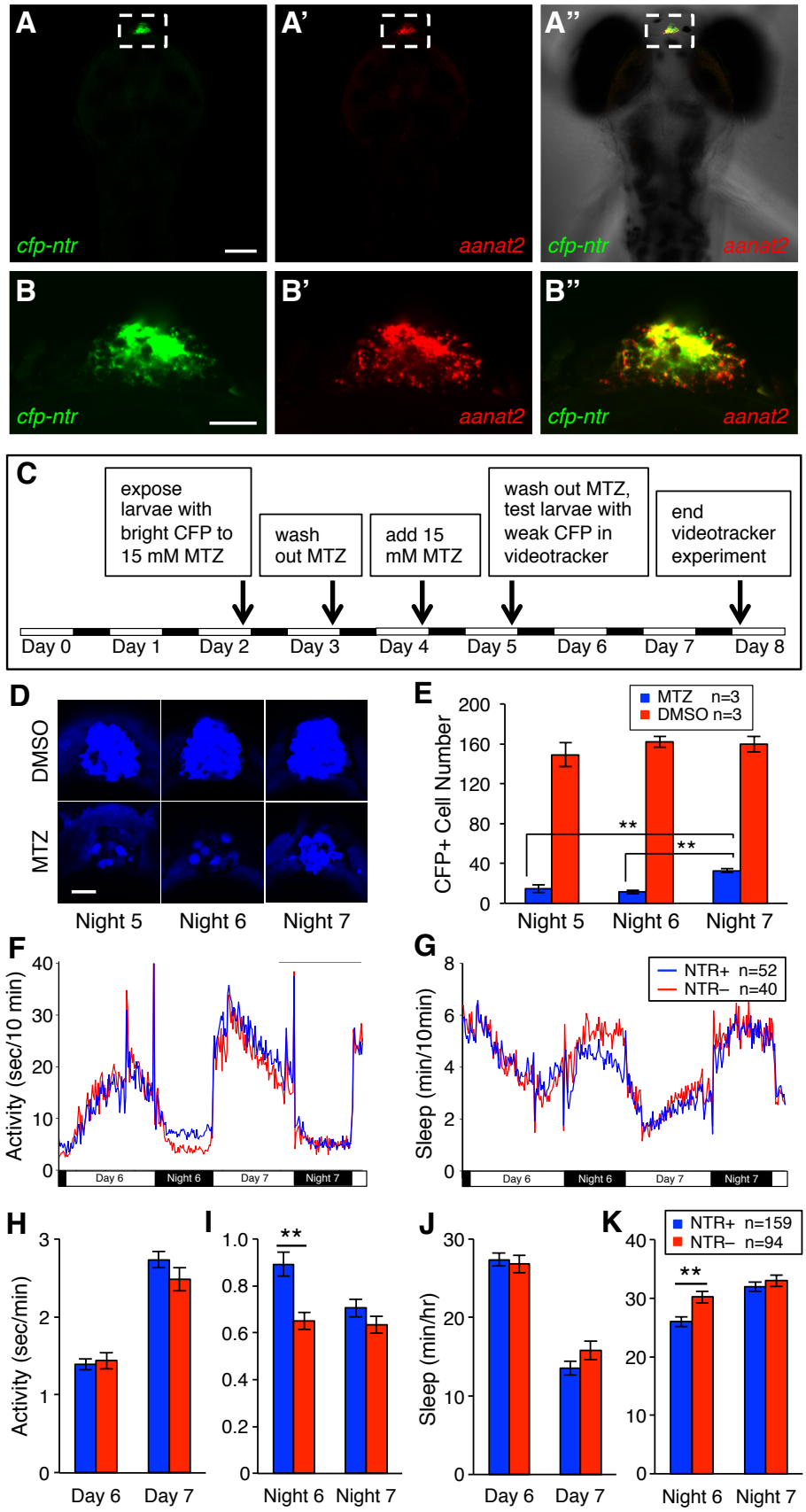

A
A'
A''
cfp-ntr
aanat2
B
B'
B''
C
expose larvae with bright CFP to 15 mM MTZ
wash out MTZ
add 15 mM MTZ
wash out MTZ, test larvae with weak CFP in videotracker
end videotracker experiment
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
D
DMSO
MTZ
Night 5
Night 6
Night 7
E
MTZ n=3
DMSO n=3
CFP+ Cell Number
**
F
Activity (sec/10 min)
Day 6
Night 6
Day 7
Night 7
G
NTR+ n=52
NTR– n=40
Sleep (min/10min)
H
Activity (sec/min)
I
J
Sleep (min/hr)
K
NTR+ n=159
NTR– n=94

Figure S3

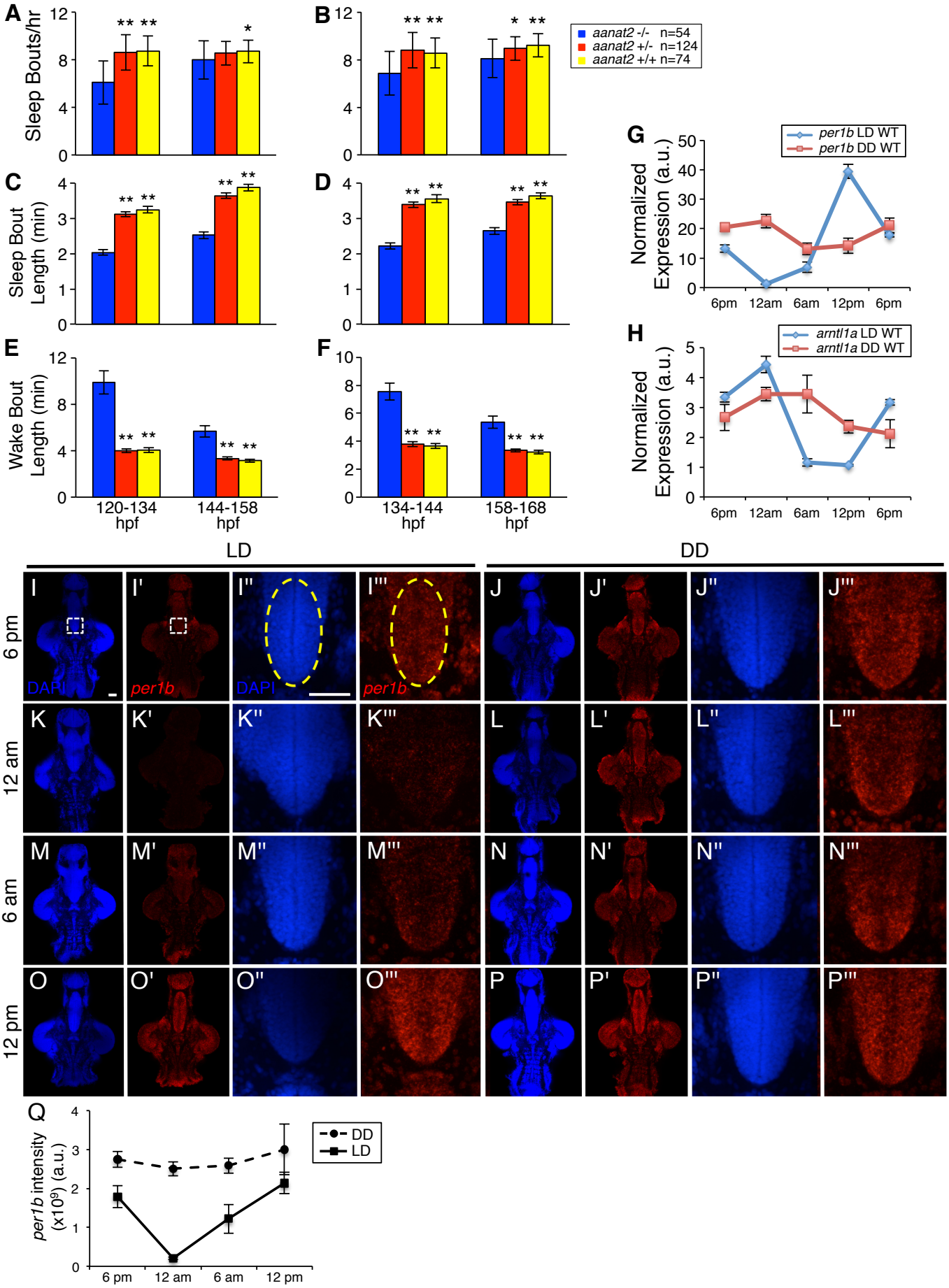
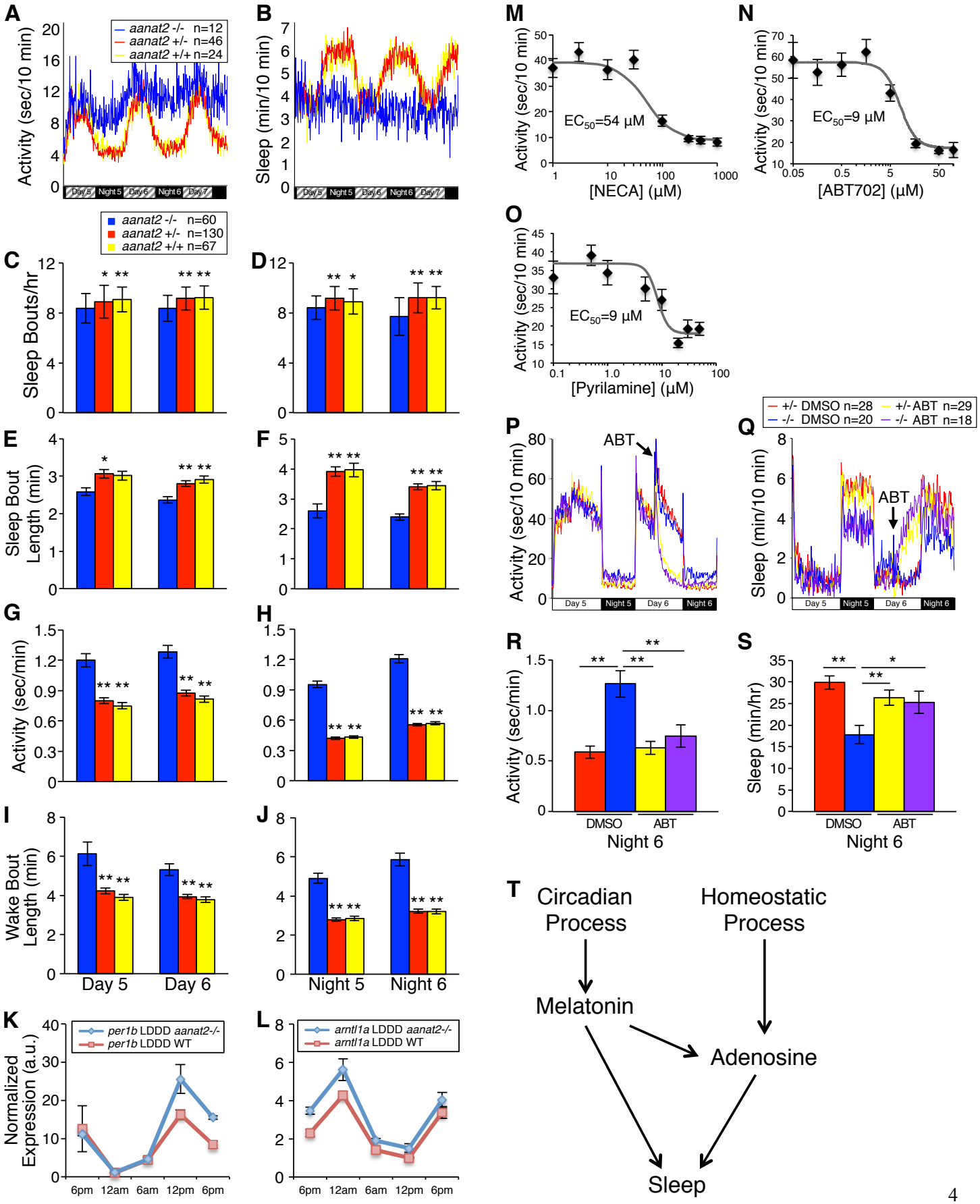

A
Sleep Bouts/hr
B
aanat2 -/- n=54
aanat2 +/- n=124
aanat2 +/+ n=74
C
Sleep Bout Length (min)
D
E
Wake Bout Length (min)
F
120-134 hpf
144-158 hpf
134-144 hpf
158-168 hpf
G
per1b LD WT
per1b DD WT
Normalized Expression (a.u.)
6pm
12am
6am
12pm
6pm
H
arntl1a LD WT
arntl1a DD WT
LD
DD
6 pm
12 am
6 am
12 pm
DAPI
per1b
Q
per1b intensity (x10⁹) (a.u.)
DD
LD
6 pm
12 am
6 am
12 pm

Figure S4


A
aanat2 -/- n=12
aanat2 +/- n=46
aanat2 +/+ n=24
Activity (sec/10 min)
Day 5
Night 5
Day 6
Night 6
Day 7
B
Sleep (min/10 min)
aanat2 -/- n=60
aanat2 +/- n=130
aanat2 +/+ n=67
C
Sleep Bouts/hr
D
E
Sleep Bout Length (min)
F
G
Activity (sec/min)
H
I
Wake Bout Length (min)
Day 5
Day 6
J
Night 5
Night 6
K
Normalized Expression (a.u.)
per1b LDDD aanat2-/-
per1b LDDD WT
6pm
12am
6am
12pm
6pm
L
arntl1a LDDD aanat2-/-
arntl1a LDDD WT
M
Activity (sec/10 min)
EC50=54 μM
[NECA] (μM)
N
EC50=9 μM
[ABT702] (μM)
O
EC50=9 μM
[Pyrilamine] (μM)
P
ABT
Q
+/- DMSO n=28
-/- DMSO n=20
+/- ABT n=29
-/- ABT n=18
Sleep (min/10 min)
R
DMSO
ABT
Night 6
S
Sleep (min/hr)
T
Circadian Process
Homeostatic Process
Melatonin
Adenosine
Sleep

Supplemental Figure Legends

Figure S1. *aanat* mutant sequences and the effects of *aanat2* mutation on melatonin levels and behavior in LD (Related to Figure 1). (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 representative 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 bar=20 µm. (E-L) *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 (F), shorter sleep bouts (H), longer sleep latency (J), and longer wake bouts (L) at night, but no phenotype during the day (E, G, I, K). Data is combined from three experiments. Median ± median absolute deviation (MAD) (E, F) and mean ± SEM (G-L) are shown. n, number of larvae analyzed. *, $p<0.05$; **, $p<0.01$

compared to *aanat2*$^{-/-}$ by Steel-Dwass (E, F) or Dunnett's test (G-L). (M) 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. (N) 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.

Figure S2. Depletion of *aanat2*-expressing pineal gland cells decreases sleep at night (Related to Figure 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.

Figure S3. Reduced sleep in *aanat2*$^{-/-}$ zebrafish does not require an entrained circadian rhythm (Related to Figure 2). (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 ($p<1x10^{-6}$ 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.

Figure S4. Melatonin is required for the circadian regulation of sleep (Related to Figure 4). (A-J) Graphs are based on data shown in Figures 4A-4C. (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. (M-O) Dose response curves for NECA (M), ABT702 (N) and pyrilamine (O). 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 EC_{50} concentration for behavioral experiments. (P-S) Larvae were treated with either vehicle or 30 μM ABT702 starting on day 6. On night 6, vehicle-treated *aanat2*$^{-/-}$ larvae are more active (P, R) and sleep less (Q, S) than vehicle-treated *aanat2*$^{+/-}$ larvae. In contrast, ABT702-treated *aanat2*$^{-/-}$ larvae exhibit the same amount of activity (P, R) and sleep (Q, S) 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. (T) 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.

Supplemental Experimental Procedures

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)* 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 28.5^{O}C 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 $CaCl_2$, 0.33 mM $MgSO_4$, 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 28.5^{O}C. 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 EC_{50} 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, The Mathworks, 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* transgene (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 $CaCl_2$, and 2.4 mM $NaHCO_3$, 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. *Tg(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, *Tg(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 prevent photo-degradation of melatonin. 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 4°C. 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 4°C. 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 highest ΔCt value for each gene of the LD samples or the WT LDDD samples (for Figures S3G-S3H and S4K-S4L, respectively).

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