Supporting Information

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SI Materials and Methods

Fish Maintenance and Generation of Transgenic Lines. Animals were kept under 14-h light/10-h dark (14:10 LD; ZT 0, 9 AM) cycle at 28 °C, under optimal maintenance conditions in accordance with the animal protocol approved by Stanford University. The previously isolated 2-kb promoter of zebrafish *hcrt* (1) was subcloned upstream of enhanced green fluorescent protein (*hcrt:EGFP*) and was used for transgenesis using the tol2 transposable technique (2) and five stable transgenic lines expressing EGFP specifically in the HCRT cells were isolated. These five independent lines showed consistently the same expression and projection patterns, the brightest was used for imaging experiments. The specificity of the expression pattern was controlled using a double fluorescent in situ hybridization and immunostaining between the endogenous *hcrt* mRNA and EGFP protein, see Fig. S1.

In Situ Hybridization and Immunohistochemistry. Larvae (2dpf) and brains dissected from 6-month-old adult fish were fixed in 4% PFA over 48 h at 4 °C. All samples were first dehydrated in 100% methanol and stored at −20 °C. Before experimentation, brains and larvae were rehydrated in PBS. Adult brains were embedded in 2.5% agarose and sectioned with a vibratome (series 1000, Sectioning System). Transverse sections were then processed and stained as free-floating slices. ISH was performed following standard protocols. Digoxigenin or fluorescein labeled antisense riboprobes for *hcrtr*, *aanat2*, *egfp*, *gad65*, *gad67*, *vglut1*, *vglut2a*, *vglut2b*, *mtr1b1*, and *mtr1b2* were transcribed in vitro using standard reagents (Roche Molecular Biochemicals). ISH were revealed with colorimetric (BM purple, INT-BCIP) or fluorescent staining (TSA green, Fast red). Immunohistochemistry was performed following standard protocols. Adult brains were fixed in 4% PFA overnight at 4 °C and then cut as described above. Slices were blocked with 0.4% Triton X-100 and 20% normal goat serum in PBS for 1 h at room temperature. After blocking, adult brain slices were incubated in primary antibody rabbit polyclonal anti-GFP (Torrey Pines Biolabs, TP401) antibody (1:100 dilution) in block buffer overnight at 4 °C. Slices were again washed in PBS + 0.4% Triton X-100 and blocked for 1 h. Anti-EGFP antibodies were then detected using a secondary goat-anti-rabbit Alexa Fluor 488 IgG (H+L) antibody, highly cross-adsorbed (2 mg/mL, Invitrogen, Molecular Probes). The incubation (dilution 1:400) was done for 2 h and followed by washes in PBS.

Two-Photon and Confocal Imaging. To visualize HCRT axonal and dendritic processes, living *hcrt:EGFP* transgenic larvae were mounted in low melting point agarose and imaged by two photon microscope. Imaging was performed using a 63×/0.9 numerical aperture water-immersion objective (Zeiss), on a custom-made two photon laser-scanning microscope, tuned to 850 nm for excitation of EGFP. Adult sections fluorescence images were obtained using a Zeiss LSM 510 META laser scanning confocal microscope.

Pineal Gland Perfusion System and Radioimmuno Assay (RIA) of Melatonin. Adult zebrafish were anesthetized with Tricaine (3-amino benzoic acidethylester; Sigma), pineal glands were removed and cultured in a flow-through system as described in refs. 3 and 4. Each individual pineal gland was placed into a glass tube, which is situated in a lightproof box, and perfused continuously with medium (1 mL/h) (Fig. S2). The culture medium was MEM (Sigma) supplemented with 2 mM L-glutamine, 0.1 mM L-tryptophan, 0.02 M sodium bicarbonate, penicillin (100,000 U/L), and streptomycin (100 mg/L) (Biological Industries Ltd). Fractions (0.5 mL) of medium were collected at 30-min intervals. Pineal glands were exposed to two LD cycles followed by DD. To test the effect of HCRT on melatonin production, zebrafish HCRT-1 (10^{-6} M) or HCRT-free MEM media were added to the perfusion tubes through a T-shape valve (see Fig. S2, modified from ref. 4) for 2 h. Melatonin in media was determined by RIA (RIA) using melatonin antibodies (Stockgrand Ltd.) according to the manufacturers' protocol and as described in ref. 4.

The effect of HCRT application on melatonin production was analyzed by comparing melatonin levels during the treatment to those in the hours before treatment (see *Statistics*). To account for variation in the basal levels of melatonin secretion from individual pineal glands (3), melatonin levels secreted by each pineal gland were normalized by dividing the absolute levels by the average night-time levels (before HCRT treatments, ZT 17–21).

Behavior Assays and Pharmacological Treatment. Locomotor activity of larvae was monitored using an automated video-tracking system (Videotrack; ViewPoint Life Sciences) and the movement of each larva was recorded using Videotrack quantization mode. On the fifth day of development, individual larvae were placed in 24-well plates that allowed for a relatively high volume of water for swimming and prevented the larvae from interfering with each other. The detection threshold parameter in the Videotrack quantization was set to 30. Each experiment consisted of *hcrtr*−/− and wild-type sibling larvae placed alternately in the different wells to avoid potential small differences in their environment and camera sensitivity. Larvae from each genotype were monitored in drug-free condition and under melatonin (0.001 mM), pentobarbital (0.01 mM, 0.1 mM), Flurazepam (0.1 mM, 1 mM), Mepyramine (1 mM, 10 mM), and Clonidine (0.1 mM, 1 mM) administration. The choice of hypnotics and respective sedative doses were based on Dr. Zhdanova pioneering work (5) and our own study of the effects of sedative hypnotics commonly used in humans on zebrafish (6). Locomotor activity was monitored for 24 h under dim light (LL, <10 lux) starting at ZT 6 (Zeitgeber time). The activity was integrated every 2 h. Three to five independent experiments ($n = 10$ for each genotype in each independent experiment) were performed for each treatment.

Sleep of adult fish was monitored with AFSRS (adult fish sleep recording system) as described in ref. 7. Adult *hcrtr*−/− ($n = 12$) and wild-type siblings ($n = 12$) were raised in 14:10 LD cycle facility, then transferred to the system and monitored using an infrared beam under the same LD conditions. After 2 days of habituation, melatonin (1 μM) was added (ZT 15) and the fish were monitored for additional 2 LD cycles.

Real-Time PCR Quantification of *aanat* mRNAs in the Pineal Gland. Expression of zebrafish arylalkylamine N-acetyltransferase (*aanat2*) and orthodenticle homeobox 5 (*otx5*) in the pineal gland during the night (1:00 AM, ZT 16) was determined at the transcript level using quantitative PCR assays. Adult zebrafish raised in LD conditions (see above) were anesthetized with Tricaine, six pineal glands were collected for each genotype (*hcrtr*−/− and wild-type sibling). Total RNA was extracted using RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's instructions. Similar amount of mRNA was reverse transcribed using Oligo(dT) primer and SuperScript II RT

(Invitrogen). Transcript levels were determined by Corbett 6000 Rotor-Gene Q real-time PCR cycler using the DyNAmo Flash SYBR Green qPCR Kit (Finnzymes) following the manufacturer's instructions. Triplicate first-strand cDNA aliquots from each sample served as templates in real-time PCR. The relative quantification of *aanat2* gene expression levels was normalized against the arrhythmic and pineal specific *otx5* gene (8) and analyzed by Rotor-Gene Q software subjected to the $\Delta\Delta C_T$ method.

Statistics. In behavioral experiments, statistical comparisons were performed using two-tailed paired *t* test in adults and repeated-measure ANOVA with grouping factor, in larvae. In cultured pineal gland assay, average melatonin levels in each column during HCRT or MEM control treatment (ZT21–23) were compared to the average melatonin levels before treatment (ZT15.5–21) and the differences were determined by ANOVA with repeated measurements. In quantitative real-time PCR assays *aanat2* levels were normalized by dividing the absolute levels of each pineal gland with the average of all samples. Student's *t* test was used to showed significant differences in the expression levels of *aanat2* between the two genotypes.

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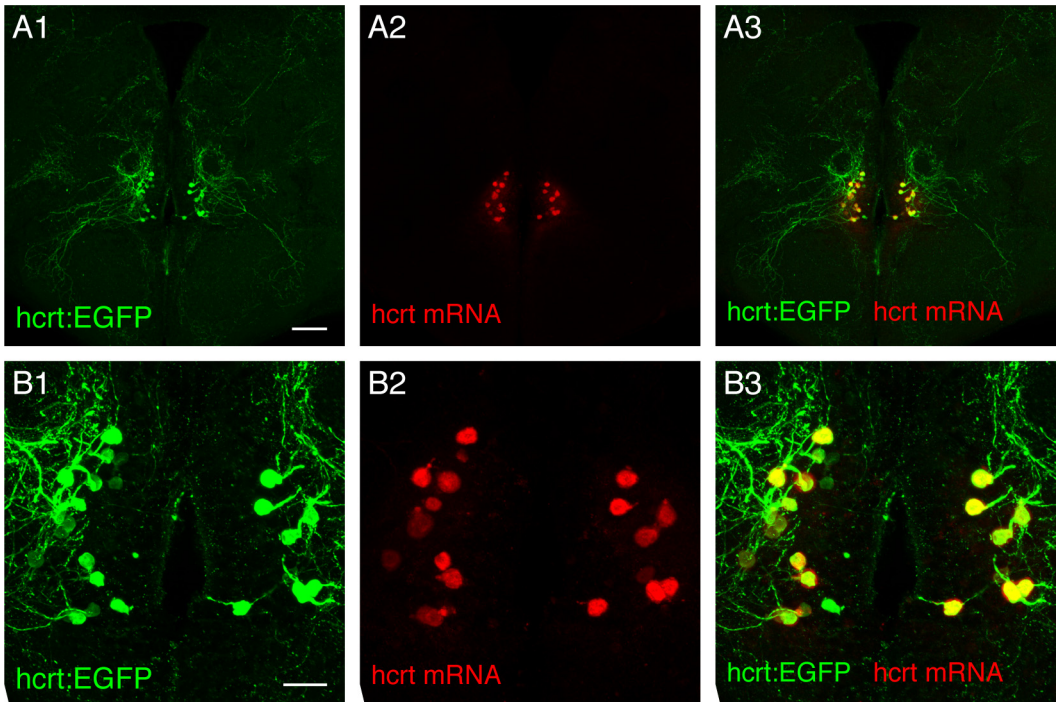

Fig. S1. (*A1–A3* and associated close-ups *B1–B3*) Double fluorescent ISH and Immunostaining between endogenous *hcrt* mRNA and EGFP protein driven by *hcrt* promoter as visualized using confocal microscopy on brain sections of adult *hcrt:EGFP* transgenic fish (reconstructed stacks of 0.5- or 1-μm sections). Note the very good colocalization demonstrating the specificity of the *hcrt* promoter fragment used in the transgenic lines. (Scale bar, 100 μm *A1–A3*; 20 μm *B1–B3*.)

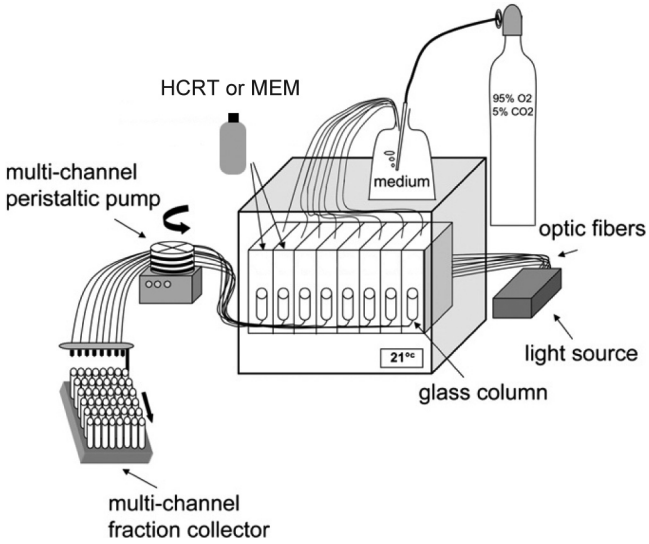

Fig. S2. A flow-through system to measure light-sensitive melatonin release from cultured zebrafish pineal glands (*SI Materials and Methods*). This illustration was modified with permission from Ziv and colleagues (2007).

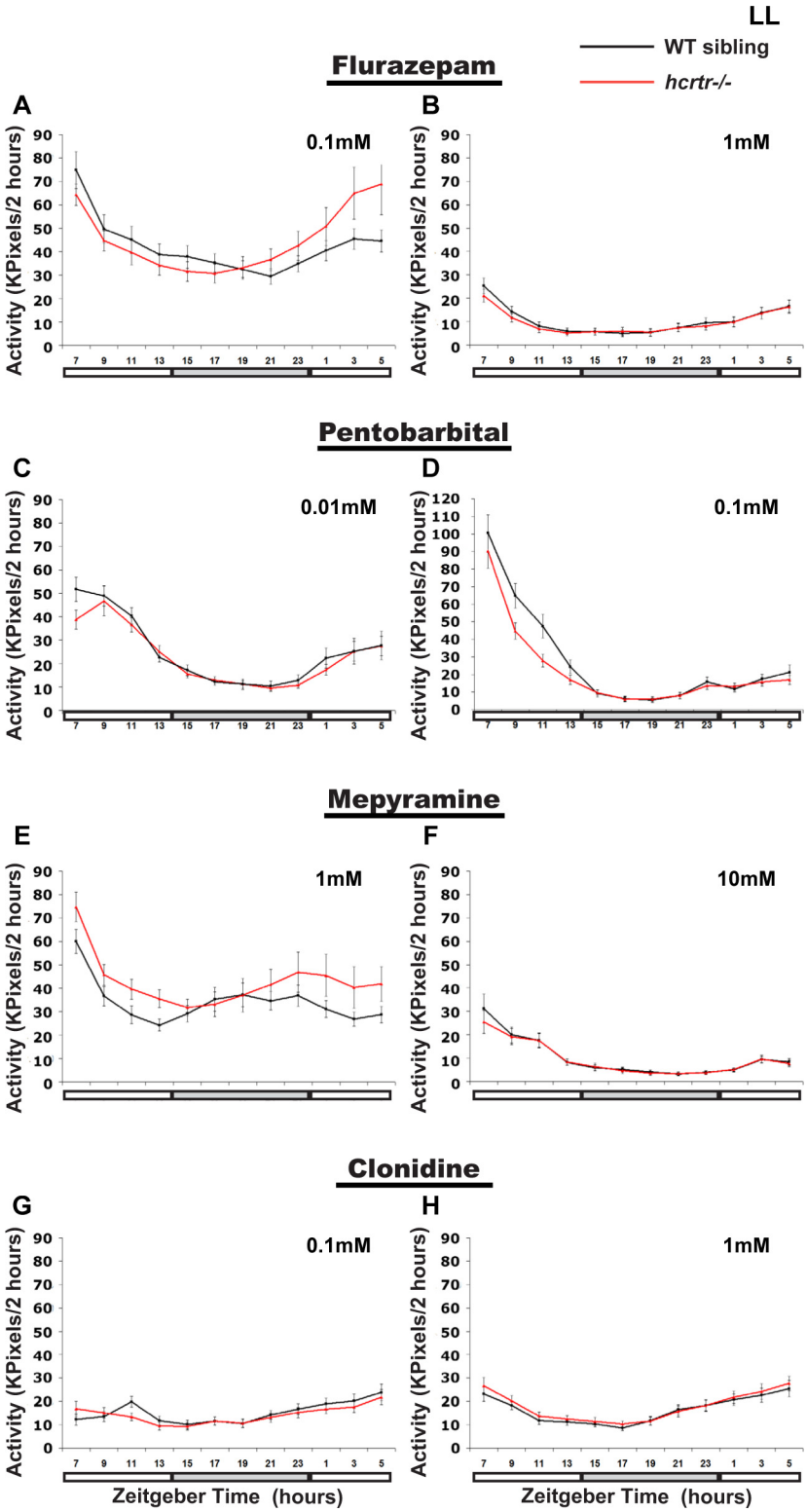

Fig. S3. *hcrtr*−/− and wild-type sibling larvae are similarly sedated by different classes of hypnotic drugs. Five dpf larvae ($n \geq 30$ for each genotype) were treated with mild and strong doses of four different classes of hypnotics: the GABAergic hypnotics benzodiazepine flurazepam (*A*) and barbiturate pentobarbital (*B*), the histaminergic H1 antagonist mepyramine (*C*), and the alpha2 adrenergic agonist clonidine (*D*). Drugs and doses were based on our previous study of the effects of sedative hypnotics commonly used in humans on zebrafish (*SI Materials and Methods*). Larvae activity was monitored over 24 h under constant dim light conditions (LL), gray bars represent the subjective night period. Note the similar response of both genotypes to the different treatments in contrast to the discriminative effect of melatonin shown in Fig. 6*A*.

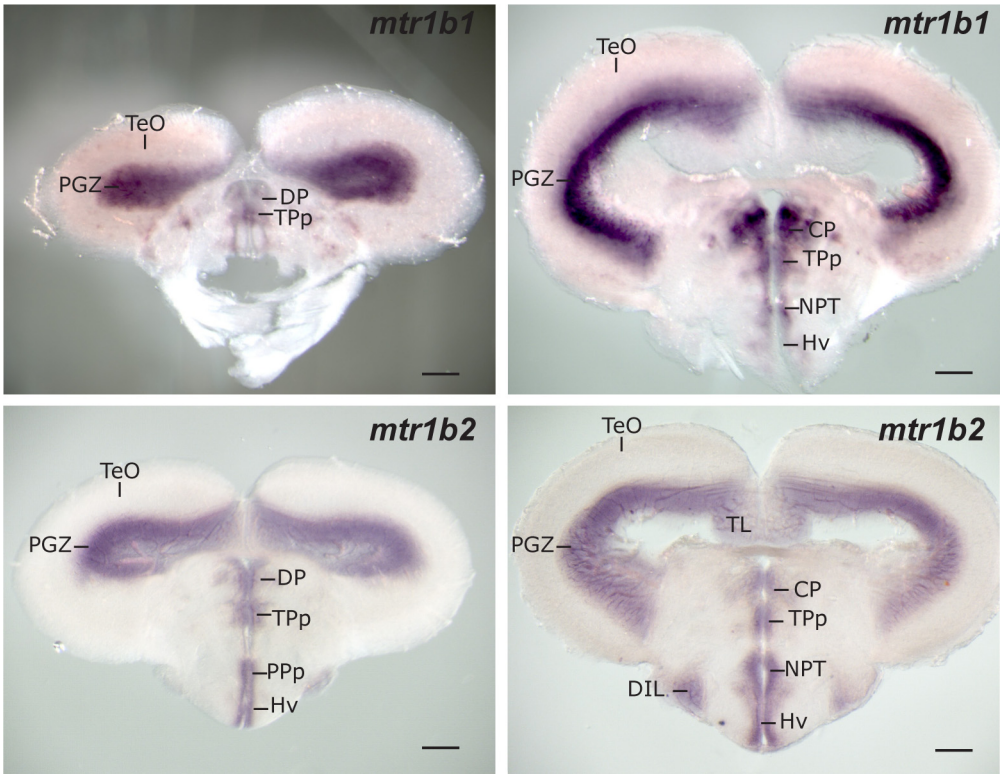

Fig. S4. melatonin receptor 1b1 (NM_131394) and 1b2 (NM_131395) mRNA ISH patterns in adult brain sections. Note the similar distribution with HCRT fibers and HCRT receptor mRNA displayed in Fig. 3. CP, central posterior thalamic nucleus; DIL, diffuse nucleus of the inferior lobe; DP, dorsal posterior thalamic nucleus; Hv, ventral zone of the periventricular hypothalamus; NPT, posterior tuberal nucleus; PGZ, periventricular gray zone of the optic tectum; PPp, parvocellular preoptic nycleus, posterior part; TeO, optic tectum; TL, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum. (Scale bar, 200 μm.)