Supplemental Figure Legends

Supplemental Figure 1. Comparison of mammalian and teleost Hcrt and Hcrt Receptor proteins. (A) Black shaded residues are identical to human Hcrt. Grey shaded residues are identical to Fugu Hcrt but are not conserved in human. Red line indicates Hcrt1 peptide, blue line indicates Hcrt2 peptide, and green lines indicate consensus peptide cleavage sequences. The mature zebrafish Hcrt2 peptide is 54% identical to its human homolog. The mature zebrafish Hcrt1 peptide is 45% identical to its human homolog, not including the 16 amino acid spacer sequence that is located in a predicted loop of a helix-loop-helix motif (Takai et al., 2006). As in mammals (de Lecea et al., 1998; Sakurai et al., 1998; Sakurai et al., 1999) and in agreement with a recent report (Faraco et al., 2006), we found that zebrafish Hcrt consists of a short first exon and a longer second exon. The region of each protein that is encoded by the first exon is outlined in purple. (B) Hcrt phylogenetic tree. (C) Black shaded residues are identical to human Hcrt Receptor 2. Grey shaded residues are identical to Fugu Hcrt Receptor but are not conserved in human Hcrt Receptor 2. (D) Hcrt Receptor phylogenetic tree. Fugu and Tetraodon sequences were identified using Ensembl databases. Sequence analysis was performed using MegAlign (DNAstar).

Supplemental Figure 2. Hcrt neurons project to Tyrosine Hydroxylase (TH)-expressing neurons in the diencephalon and locus coeruleus. (A) Ventral view of a 120 hours post-fertilization (hpf) *hcrt*-EGFP transgenic brain shows dense *hcrt*-EGFP projections that are in close apposition to projections from TH-expressing diencephalic dopaminergic neurons (B; Ma, 1997; Holzschuh et al., 2001; Kaslin and Panula, 2001;

McLean and Fetcho, 2004). *hcrt*-EFGP projections also colocalize with dense TH-expressing projections that originate from the locus coeruleus (C; Ma, 1997; Guo et al., 1999; McLean and Fetcho, 2004). Colocalization of *hcrt*-EGFP and TH projections occurs 5-10 μm ventral to the locus coeruleus soma, which are not visible in this projection. Mammalian dopaminergic neurons and noradrenergic neurons of the locus coeruleus also receive dense Hcrt projections (Peyron et al., 1998; Date et al., 1999; Hagan et al., 1999; Horvath et al., 1999; Nambu et al., 1999; Nakamura et al., 2000) and express Hcrt receptors (Trivedi et al., 1998; Bourgin et al., 2000; Greco and Shiromani, 2001; Marcus et al., 2001). Unlike mammals (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999) or adult zebrafish (Kaslin et al., 2004), we do not observe dense Hcrt neuronal projections to the raphe serotonergic or tuberomammillary histaminergic neurons in zebrafish larvae (data not shown). Scale bars, 50 μm (A); 10 μm (B, C).

Supplemental Figure 3. Time-lapse images of developing Hcrt neurons. Transient injection of the *hcrt*-EGFP transgene labels a single Hcrt neuron on the right side of the hypothalamus and two neurons on the left side of the hypothalamus. Boxed region in (A) is shown at higher magnification in (B). By 30hpf, both neurons have extensive dendritic and axonal arbors within the diencephalon (A,B), as well as axons that project caudally beyond the field of view. By 48hpf (C), the axons of all 3 neurons extend branches in the hindbrain to the locus coeruleus (arrowheads). The axons of two of the neurons have entered the spinal cord while the axon of the third neuron terminates in the hindbrain by 72hpf (arrow in D). The axon of a second neuron terminates its growth in the spinal cord

by day 6 of development (arrow in E). Ventral views are shown in (A,B). Side views are shown in (C-E). Anterior is to the left. Scale bars, 200 μm (A,C-E); 40 μm (B).

Supplemental Figure 4. Time-lapse images of developing Hcrt neurons. Transient injection of the *hcrt*-EGFP transgene labels a single Hcrt neuron on each side of the hypothalamus (A). By 48hpf, the axon of one of the neurons has grown ~400μm and terminates in the hindbrain (arrow in B-D). Between 72hpf and day 6 of development, the axon turns ventrally and forms branches in the hindbrain (arrow in D). Ventral view is shown in (A). Side views are shown in (B-D). Anterior is to the left. Scale bars, 200μm.

Supplemental Figure 5. Time-lapse images of a developing Hcrt neuron. Transient injection of the *hcrt*-EGFP transgene labels a single Hcrt neuron. By 72hpf, the axon of this neuron has terminated its growth in the spinal cord (A,C,E). Boxed regions in (A,C,E) are shown at higher magnification in (B,D,F), revealing the development of dendritic and axonal arbors within the diencephalon. Anterior is to the left. Scale bars, 200 μm (A,C,E); 40 μm (B,D,F).

Supplemental Figure 6. Time-lapse images of a developing Hcrt neuron. Transient injection of the *hcrt*-EGFP transgene labels a single Hcrt neuron. Boxed region in (A) is shown at higher magnification in (B,C,D). By day 4 of development, the axon of this neuron has extended a branch in the hindbrain to the locus coeruleus (arrowhead in A), and has terminated its growth in the spinal cord (arrow in A, data not shown). This

neuron extends an arbor towards the midline by day 8 (asterisk in C) that is retracted by day 11 (D). Dorsal views are shown. Scale bars, 200 μm (A); 40 μm (B-D).

Supplemental Figure 7. Heat shock induces ubiquitous *hcrt* transcription but only generates mature Hcrt1 peptide in a subset of neurons in the central nervous system. HS-Hcrt larvae were heat shocked for 1 hour at 24hpf (B,D) or 120hpf (F,H). Embryos and larvae were either fixed 1 hour post-heat shock for *hcrt* in situ hybridization (A,B,E,F) or 8 hours post-heat shock for Hcrt1 peptide antibody labeling (C,D,G,H). While *hcrt* mRNA is ubiquitously produced in heat shocked samples (B,F), Hcrt1 peptide is only observed in a subset of neurons in the spinal cord (D) and brain (D,H) that includes the neurons that express endogenous Hcrt. Endogenous *hcrt* mRNA is not detected in non-heat shocked samples due to the short in situ hybridization development time that is sufficient to detect heat shock-induced *hcrt*. Endogenous Hcrt1 peptide is difficult to observe at the magnification shown in (C). Dark spots in (E) are pigment cells. Anterior is to the left. Side views are shown in A-D. Ventral views of brains are shown in (E-H). Scale bars, 100 μm.

Supplemental Figure 8. WT larvae exhibit low levels of locomotor activity in both constant dark and constant light conditions. Each data point represents the average seconds of locomotor activity every 10 minutes for the indicated number of WT larvae. Embryos were exposed to light for 2-3 hours following fertilization, and were then either raised in constant dark (DD) (A) or constant light (LL) (B). Behavioral recording was initiated on day 4 of development. (A) In DD conditions, larvae have locomotor activity

levels similar to those observed at night in larvae maintained in a 14:10 hour light:dark cycle (LD). (B) In LL conditions, larvae have locomotor activity levels that are higher than those observed in DD or at night in LD, but lower than those observed during the day in LD. Oscillations in locomotor activity levels are not observed for individual larva under either DD or LL conditions (data not shown).

Supplemental Figure 9. Hcrt overexpression dramatically consolidates active states and reduces rest in constant light conditions. (A) Hcrt overexpression increases locomotor activity in constant light (LL) conditions. Each data point represents the average seconds of locomotor activity every 10 minutes for the indicated number of larvae of each genotype. Embryos were exposed to constant light from birth. Behavioral recording was initiated on day 4 of development and larvae were heat shocked for 1 hour on day 5 (arrow). Under LL conditions, WT larvae exhibit moderate activity levels with little oscillation in activity. Hcrt-overexpressing larvae are slightly more active than WT larvae before heat shock, but become significantly more active following heat shock. The small oscillation in locomotor activity following heat shock is likely induced by either the heat shock itself or handling of the 96-well plate as it is transferred to the 37ºC waterbath, as has previously been observed (Hurd and Cahill, 2002). (B-E) Each bar represents the mean +/- SEM of 44 HS-Hcrt or 34 WT larvae. Hcrt overexpression dramatically increases active bout length (B), decreases rest bout length (C), decreases total time at rest (D), and decreases the number of rest bouts (E) in LL conditions (*, $p<0.05$, **, $p<0.01$ by two-tailed Student's t-test). (F) Hcrt overexpression decreases rest in the entire larval population in LL conditions. The graph represents the distribution of

total rest times for HS-Hcrt and WT larvae 12-24 hours post-heat shock. (G) Pie charts represent the percentage of time spent in each state and arrows represent the frequencies of transitions between states during the 12 hours following the heat shock. HS-Hcrt larvae in the inactive and low active states are more likely to transition to the high active state than WT larvae, as represented by thicker arrows. For example, following heat shock, HS-Hcrt larvae spent 90% of their time in the high active state vs. 61% for WT, and the frequency that HS-Hcrt larvae transition from the inactive to the high active state is 46% vs. 29% for WT. Frequency values in bold are significantly different between HS-Hcrt and WT larvae ($p < 0.05$ by two-tailed Student's t-test). State transition frequencies do not add up to 1 because larvae often remain in the same state for more than 1 minute. These results, together with our experiments in light:dark cycles (Figure 3) and constant dark (Figure 7) and our arousal assay (Figure 6), demonstrate that Hcrt-overexpressing larvae exhibit the hallmark symptoms of insomnia (Zorick et al., 1981; Stepanski et al., 1988; Bonnet and Arand, 2000; Stepanski et al., 2000; Nofzinger et al., 2004; Mahowald and Schenck, 2005).

Supplemental Movie 1. WT and HS-Hcrt larvae exhibit similar levels of locomotor activity before heat shock. WT and HS-Hcrt larvae are shown on the left and right halves, respectively, of a 96-well plate in a zebrabox. The movie was recorded during the afternoon of the fifth day of development before the application of a heat shock.

Supplemental Movie 2. HS-Hcrt larvae are more active than WT larvae during the night following heat shock. A 1 hour heat shock was applied during the fifth day of

development and the movie was recorded the following night. During this 30 second movie, 46/50 HS-Hcrt larvae exhibit clear locomotor activity compared to 10/50 WT larvae.

Supplemental Movie 3. HS-Hcrt larvae are more active than WT larvae during the day following heat shock. A 1 hour heat shock was applied during the fifth day of development and the movie was recorded during the sixth day of development. Hcrt-overexpressing larvae exhibit significantly more locomotor activity than WT larvae.

Supplemental Movie 4. Sudden darkness induces locomotor activity. This movie of WT larvae was recorded at the end of the normal lights on period at the end of the sixth day of development. While many larvae exhibit some locomotor activity during lights on, almost all larvae become very active upon sudden darkness.

References for Supplemental Figure Legends

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