
Figures and figure supplements

Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish

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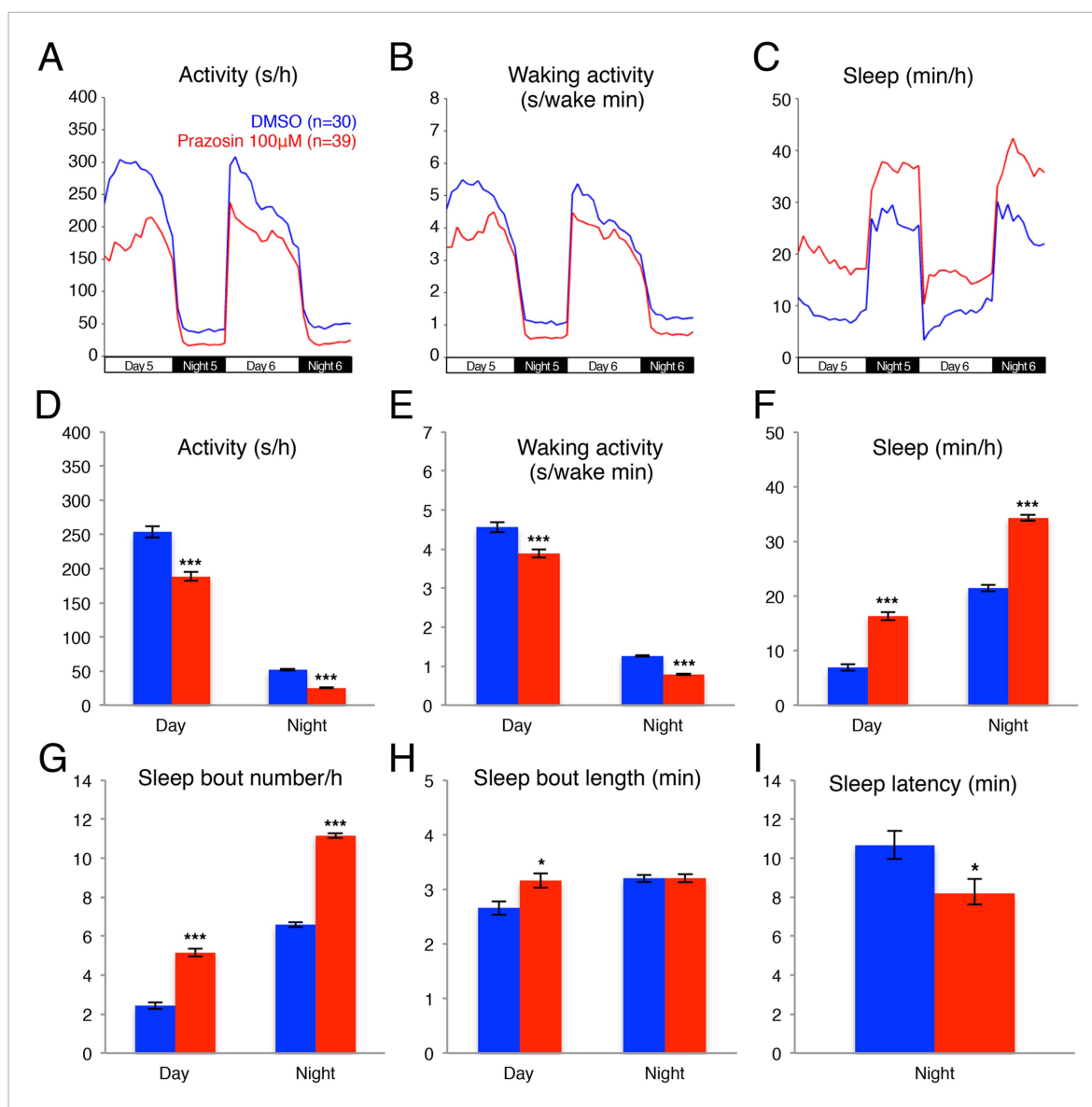


Figure 1. Prazosin treated larvae are less active and sleep more than vehicle treated controls. Representative activity (A), waking activity (amount of locomotor activity while awake) (B) and sleep (C) traces of vehicle (blue) and prazosin (red) treated zebrafish larvae. Bar graphs of activity (D), waking activity (E), sleep (F), sleep bout number (G), sleep bout length (H) and sleep latency (I) from three combined experiments (n > 180 for each condition). Bars represent mean $\pm$ s.e.m. *, $p < 0.05$ and ***, $p < 0.0001$ by one-way ANOVA.

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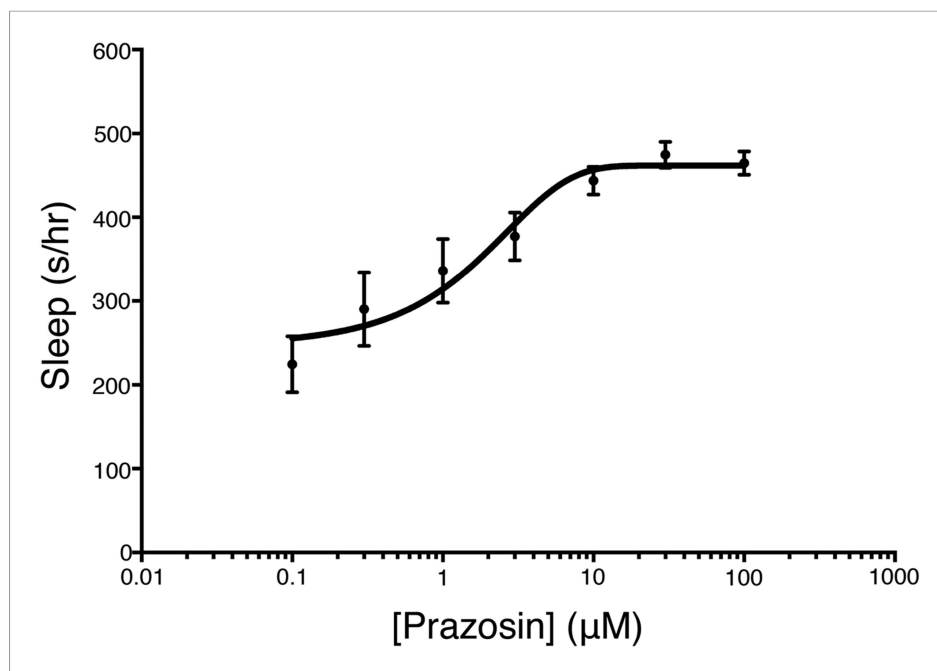


Figure 1—figure supplement 1. Prazosin night sleep dose–response curve. Larvae were treated with prazosin over range of concentrations ($n = 12$ for each concentration) on the morning of day 5 of development. Sleep was measured during night 5. Mean $\pm$ s.e.m is shown for each concentration.

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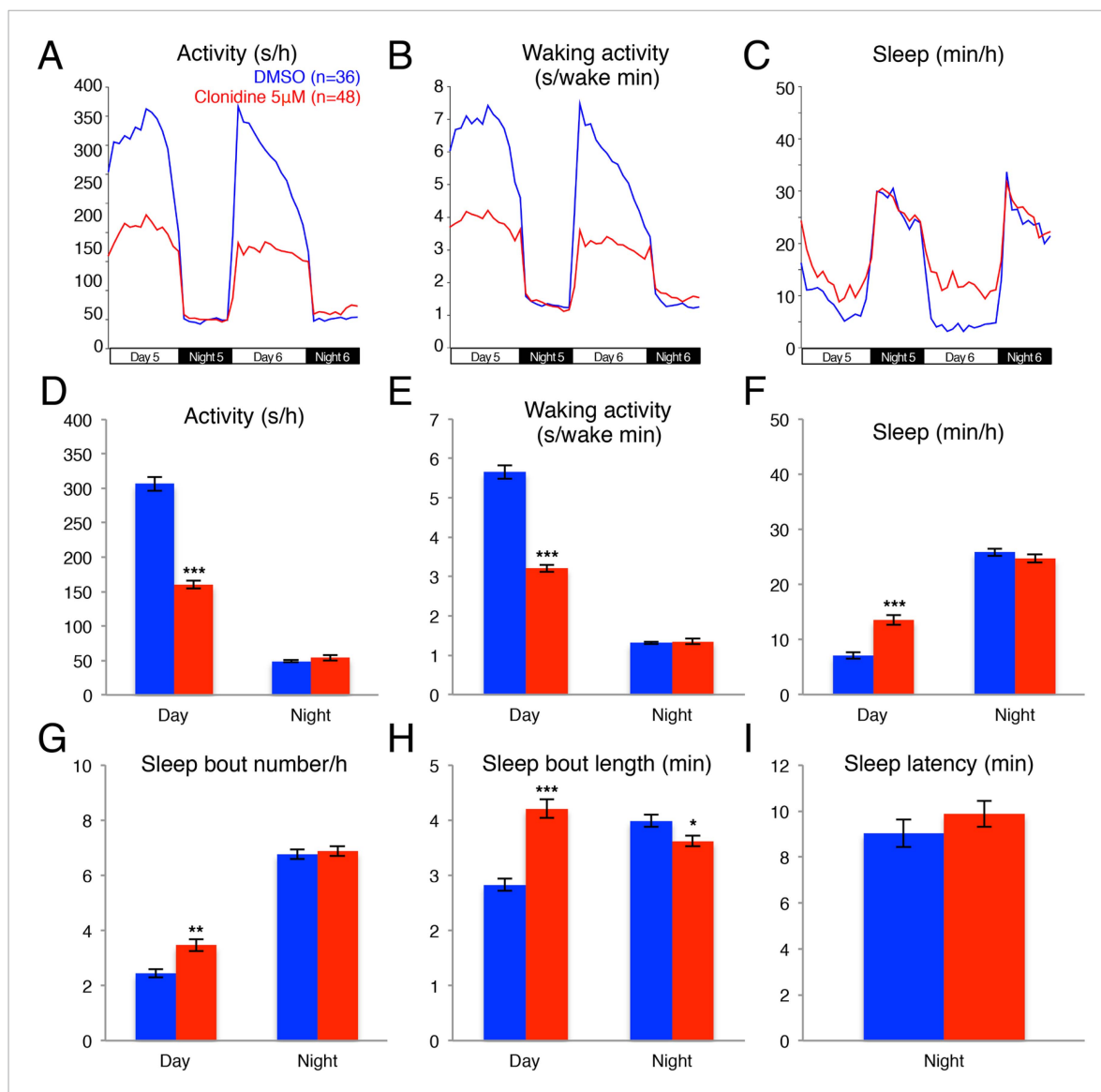


Figure 1—figure supplement 2. Clonidine treated larvae are less active and sleep more than vehicle treated controls during the day. Representative activity (A), waking activity (B) and sleep (C) traces of vehicle (blue) and clonidine (red) treated zebrafish larvae. Bar graphs of activity (D), waking activity (E), sleep (F), sleep bout number (G), sleep bout length (H) and sleep latency (I) from two combined experiments (n > 140 for each condition). Bars represent mean $\pm$ s.e.m. *, p < 0.05; **, p < 0.001; ***, p < 0.0001 by one-way ANOVA.

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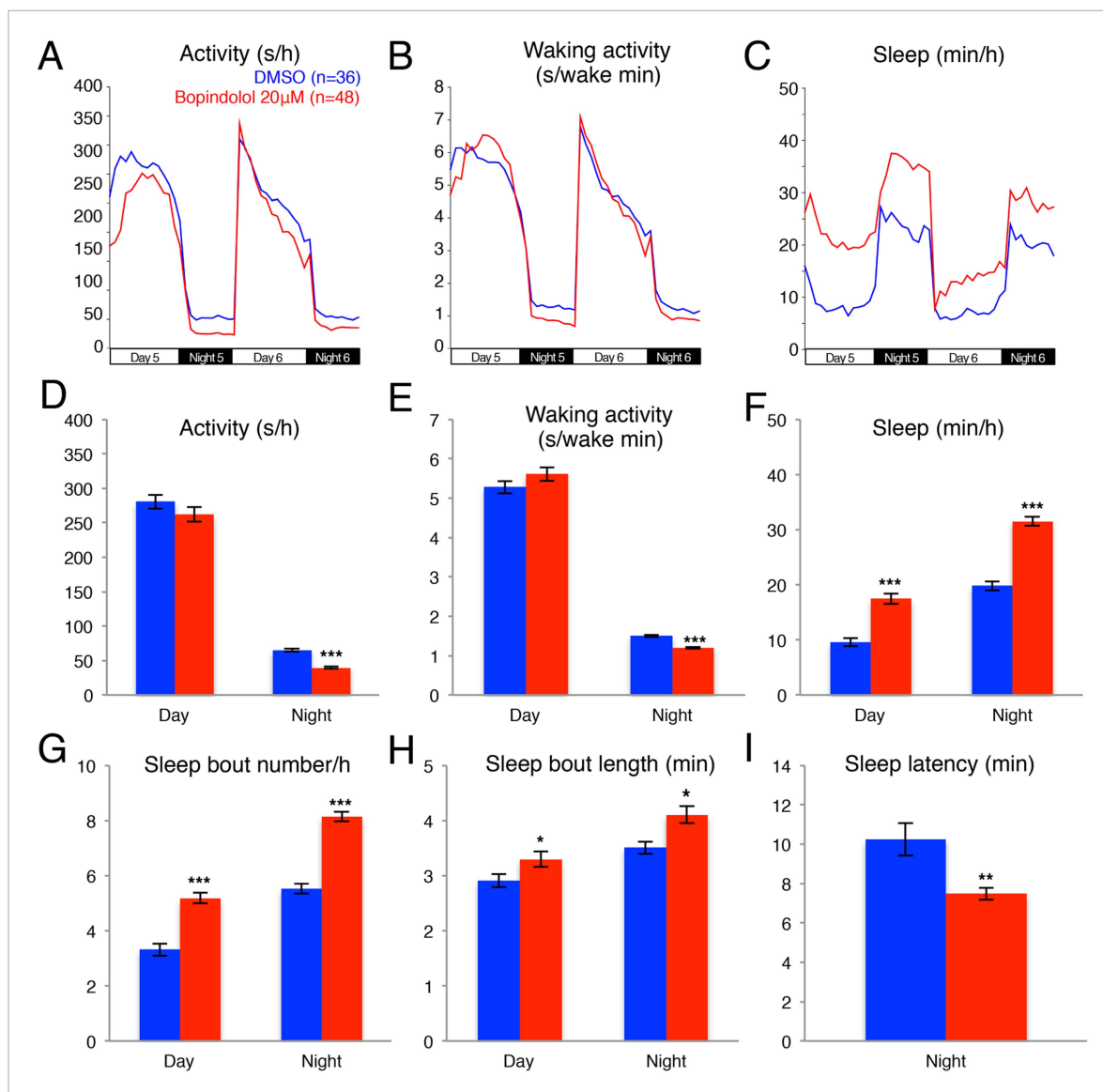


Figure 1—figure supplement 3. Bopindolol treated larvae sleep more than vehicle treated controls. Representative activity (A), waking activity (B) and sleep (C) traces of vehicle (blue) and bopindolol (red) treated zebrafish larvae. Bar graphs of activity (D), waking activity (E), sleep (F), sleep bout number (G), sleep bout length (H) and sleep latency (I) from two combined experiments (n > 140 for each condition). Bars represent mean $\pm$ s.e.m. n indicates number of larvae. *, p < 0.05; **, p < 0.001; ***, p < 0.0001 by one-way ANOVA.

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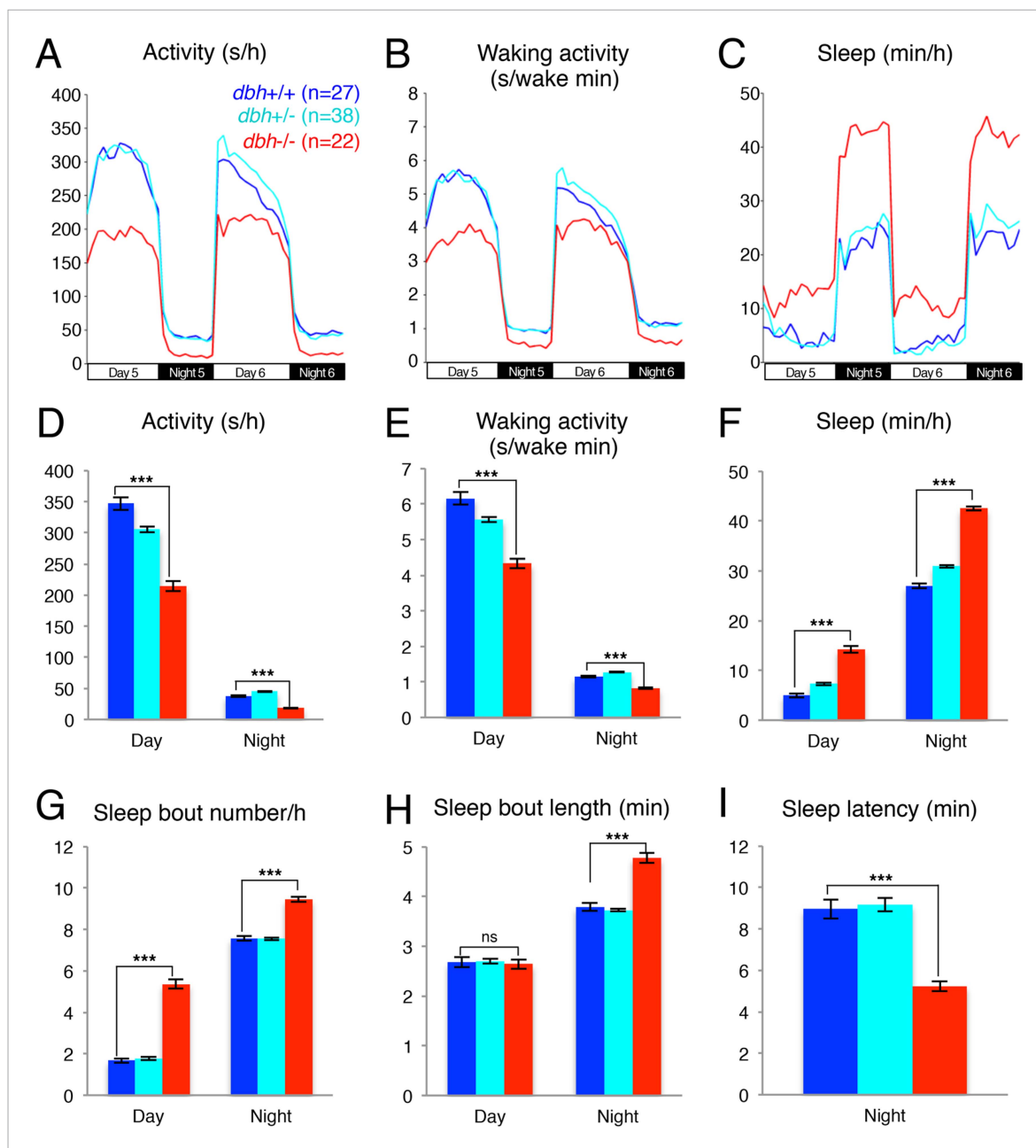


Figure 2. *dbh* mutant larvae are less active and sleep more than sibling controls. Representative activity (A), waking activity (B) and sleep (C) traces of *dbh+/+* (blue) *dbh+/-* (cyan) and *dbh-/-* (red) zebrafish larvae. Bar graphs show mean $\pm$ s.e.m. activity (D), waking activity (E), sleep (F), sleep bout number (G), sleep bout length (H) and sleep latency (I) from seven combined experiments ($n > 250$ for each genotype). n indicates number of larvae. ***, $p < 0.0001$ and ns, not significant by one-way ANOVA.

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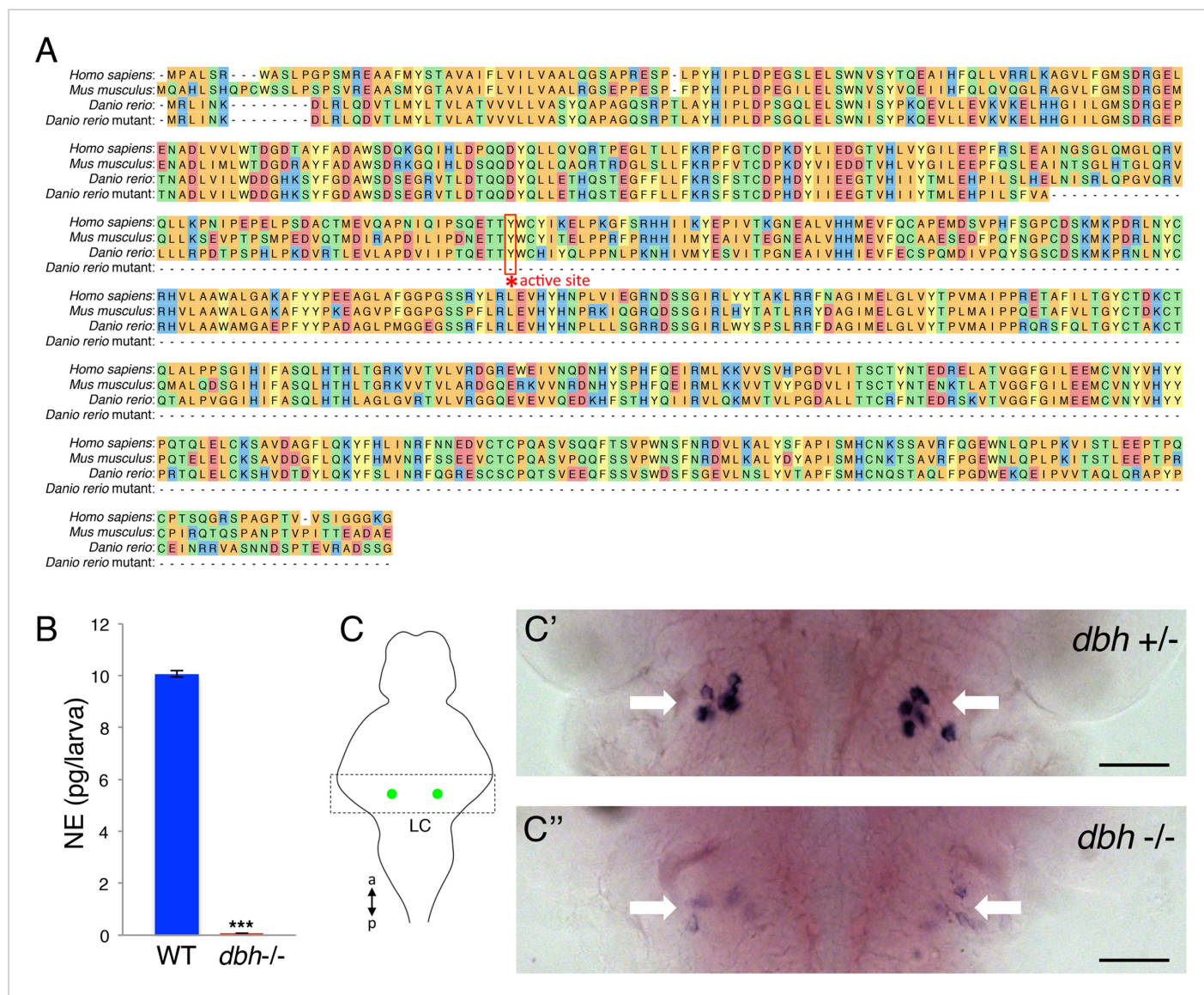


Figure 2—figure supplement 1. Verification of zebrafish *dbh* mutant. **(A)** Alignment of human, mouse, WT zebrafish and mutant zebrafish DBH amino acid sequences. The coding sequence of the mutant allele terminates before the conserved active site residue (asterisk). **(B)** ELISA quantification of NE levels in WT and *dbh*^{-/-} zebrafish larvae. Mean $\pm$ s.e.m. is shown. ***, $p < 0.0001$ by one-way ANOVA. **(C)** RNA in situ hybridization (ISH) for *dbh* in *dbh*^{+/-} (**C'**) and *dbh*^{-/-} (**C''**) sibling larvae in the LC, indicated by white arrows. Larvae were obtained from a *dbh*^{+/-} to *dbh*^{-/-} mating and were genotyped after imaging. Schematic diagram indicates the brain region (box) containing the LC (green) that is shown in the ISH images. a = anterior, p = posterior. Scale bar = 50 μ m.

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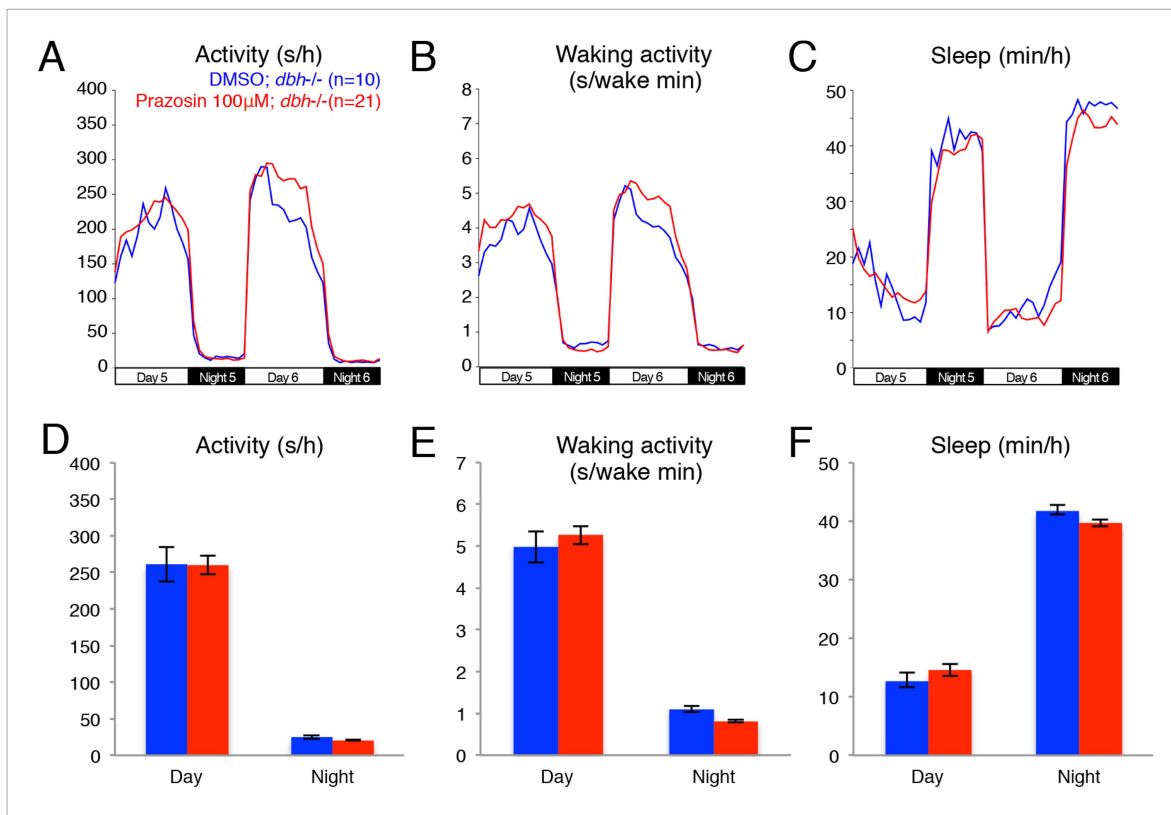


Figure 2—figure supplement 2. Treatment of *dbh*^{-/-} fish with prazosin does not alter sleep/wake behavior. Representative activity (A), waking activity (B) and sleep (C) traces of vehicle (blue) and prazosin (red) treated zebrafish *dbh*^{-/-} larvae. Bar graphs of activity (D), waking activity (E) and sleep (F) from two combined experiments (n > 45 for each condition). Bars represent mean ± s.e.m. n indicates number of larvae.

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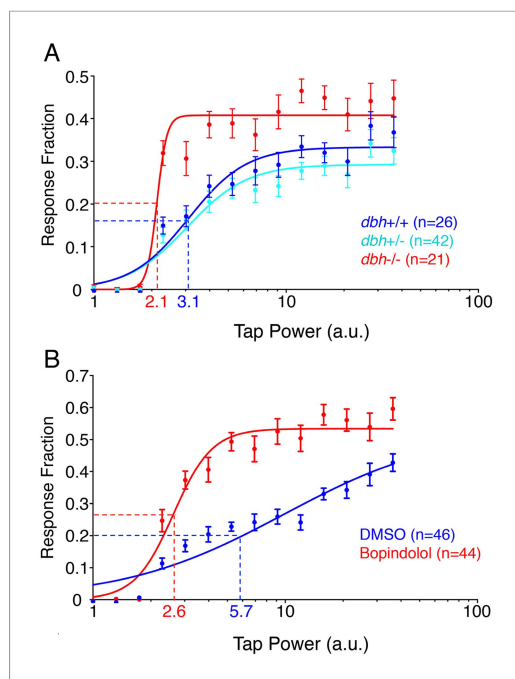


Figure 3. *dbh* mutants and bopindolol-treated WT larvae exhibit lower arousal threshold. Stimulus-response curves generated by a tapping assay for *dbh*^{-/-} and sibling control larvae (A), and bopindolol and DMSO treated WT larvae (B). Thirty trials were performed at each stimulus intensity, with a 1 min inter-trial interval. Each data point indicates mean $\pm$ s.e.m. Stimulus-response curves were constructed using the non-linear variable slope module in Prism and fitted using ordinary least squares. Dashed lines mark the ETP₅₀ values for different genotypes and drug treatments. *dbh*^{-/-} have an ETP₅₀ value of 2.1 vs 3.1 for sibling controls (32% decrease, $p < 0.0001$ by extra sum-of-squares F test) (A), and bopindolol treated animals have an ETP₅₀ value of 2.6 vs 5.7 for sibling controls (54% decrease, $p < 0.0001$ by extra sum-of-squares F test) (B). n indicates number of larvae.

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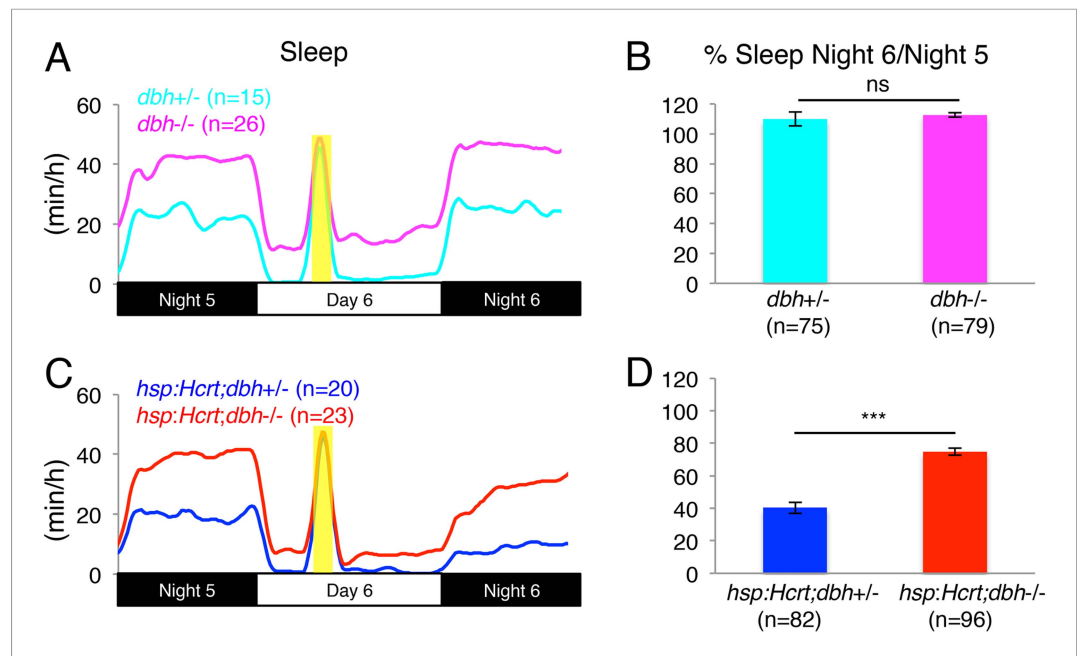


Figure 4. Reduced sleep at night due to Hcrt overexpression is suppressed in *dbh* mutants. **(A, C)** Sleep traces from a single representative experiment; yellow region indicates time of heat shock (HS) during day 6. **(B, D)** Bar graphs show mean $\pm$ s.e.m. percentage change in sleep during night 6 compared to night 5 from four combined experiments. **(B)** In the absence of the *hsp:Hcrt* transgene, HS during day 6 has no significant effect on *dbh*^{-/-} larvae compared to *dbh*^{+/+} larvae. **(D)** The reduced sleep induced by Hcrt overexpression is significantly diminished in *dbh*^{-/-} larvae compared to *dbh*^{+/+} sibling controls. n indicates number of larvae. ***, $p < 0.0001$ and ns, not significant by one-way ANOVA.

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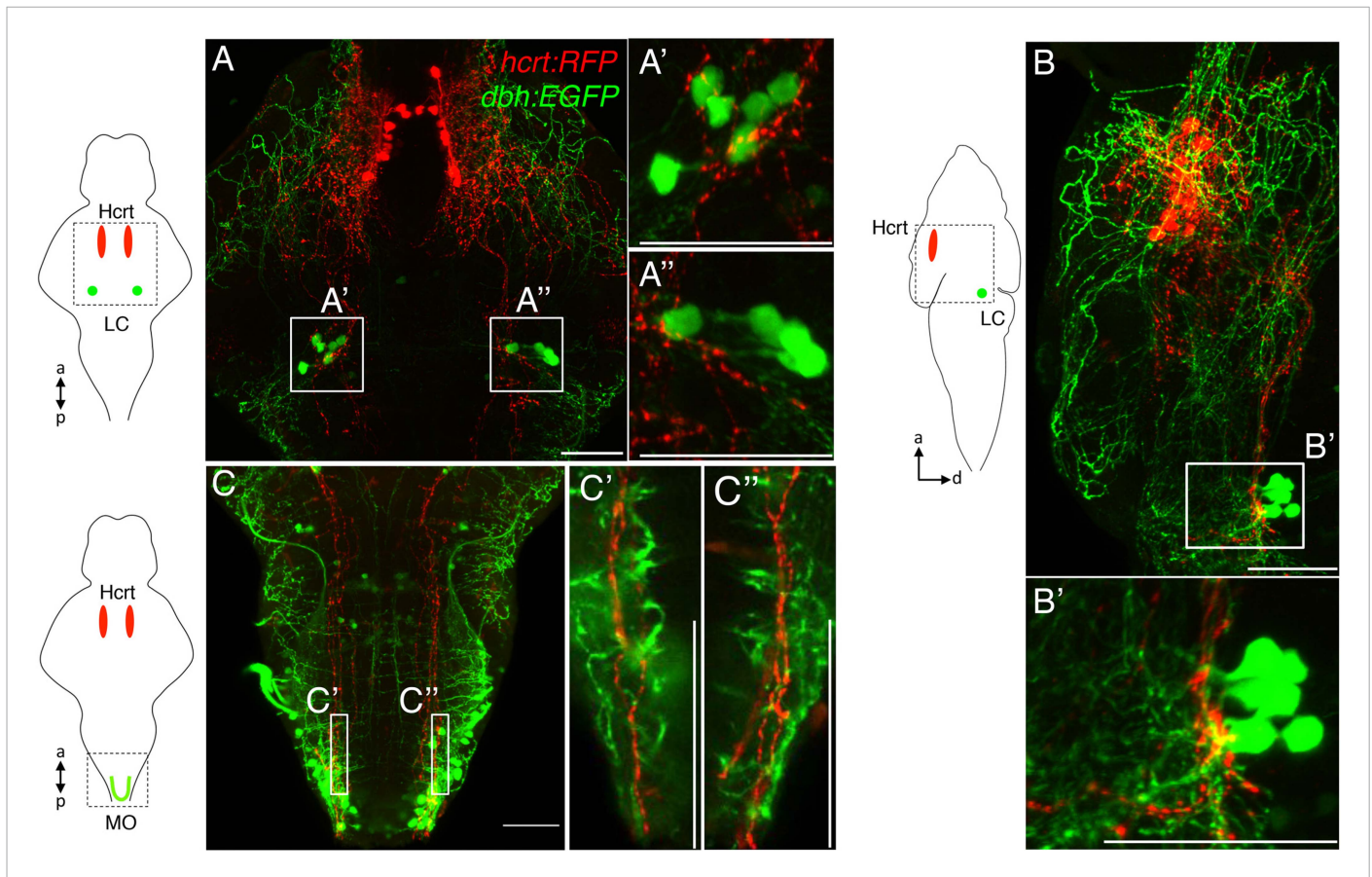


Figure 4—figure supplement 1. Hcrt neurons project to *dbh* expressing cells. Hcrt neurons (red) project to *dbh* expressing cells (green) in the LC. (A) Dorsal-ventral maximum intensity projection, (B) lateral maximal intensity projection (dorsal to the right). Boxed regions are shown at higher magnification in (A', A'', B'). (C) Projections from Hcrt neurons to *dbh*-expressing neurons in the medulla oblongata (MO). A dorsal-ventral maximum intensity projection is shown. Single optical sections of the boxed regions are shown at higher magnification in (C', C''). Schematic diagrams indicate brain regions shown in the confocal images. a = anterior, p = posterior, d = dorsal. Scale bars = 50 μm.

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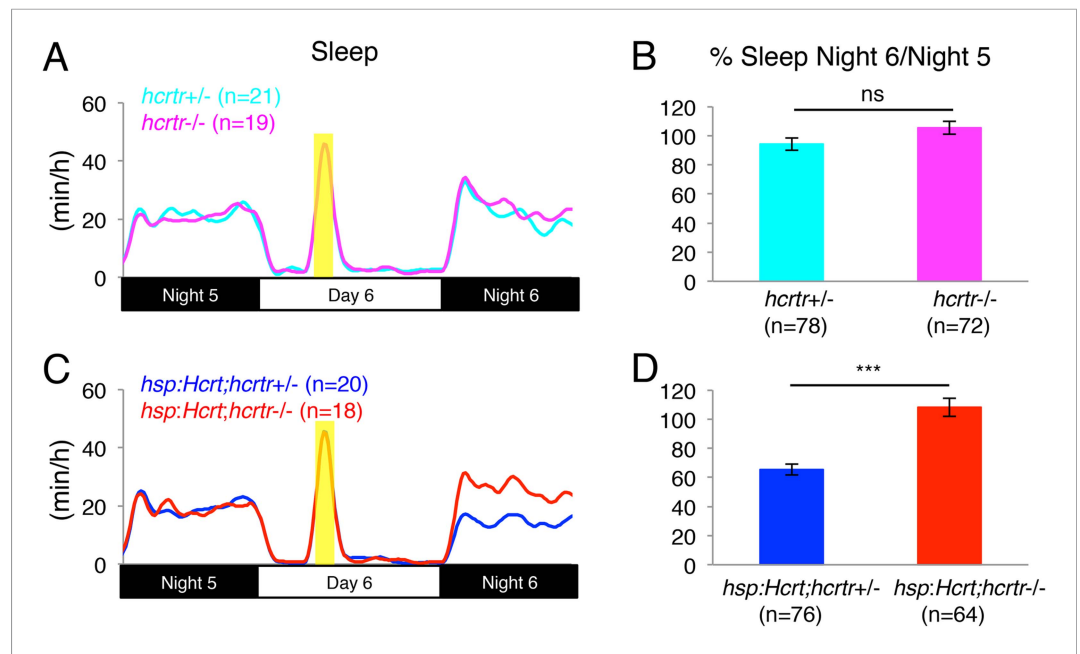


Figure 4—figure supplement 2. Reduced sleep at night following Hcrtr overexpression requires the *hcrtr*. (A, C) Sleep traces from a single representative experiment; yellow region indicates time of HS during day 6. (B, D) Bar graphs show mean $\pm$ s.e.m. percentage change in sleep during night 6 compared to night 5 from four combined experiments. (B) In the absence of the *hsp:Hcrtr* transgene, HS during day 6 has no significant effect on *hcrtr*^{-/-} larvae compared to *hcrtr*^{+/-} larvae. (D) The reduced sleep induced by Hcrtr overexpression is significantly diminished in *hcrtr*^{-/-} larvae compared to *hcrtr*^{+/-} sibling controls. n indicates number of larvae. ***, $p < 0.0001$ and ns, not significant by one-way ANOVA. We note that zebrafish *hcrtr*^{-/-} larvae have been shown to exhibit normal baseline locomotor activity and sleep levels (Appelbaum et al., 2009), similar to mouse *hcrtr* and *hcrtr* receptor 2 mutants (Willie et al., 2003; Mochizuki et al., 2011).

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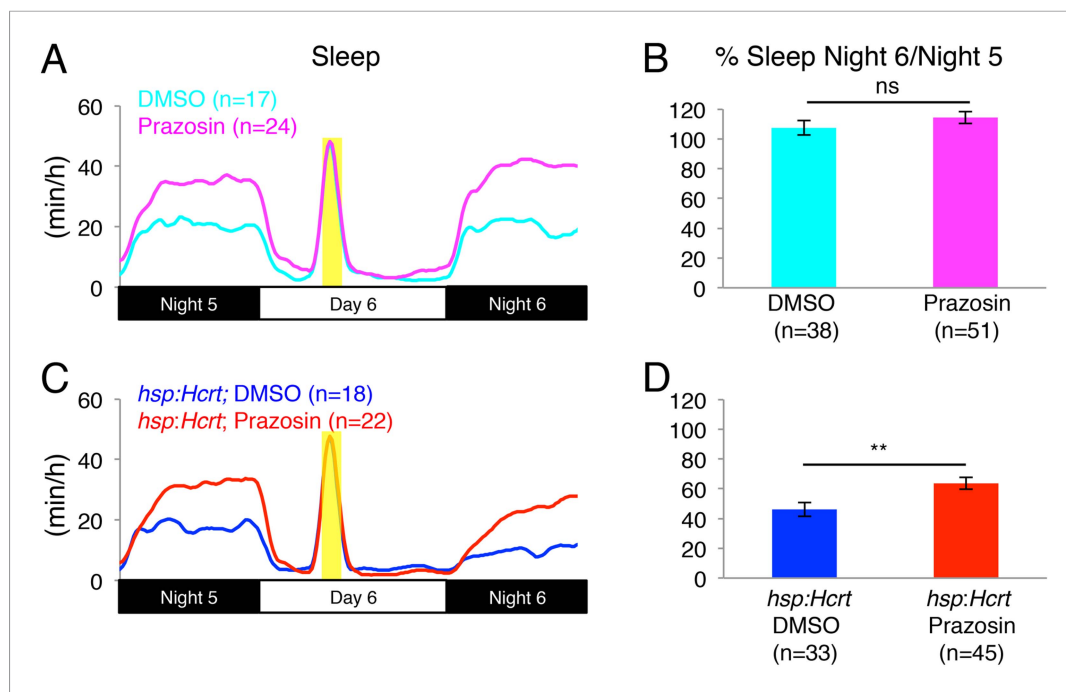


Figure 4—figure supplement 3. Reduced sleep at night due to Hcrt overexpression is suppressed by prazosin. (A, C) Sleep traces from a single representative experiment; yellow region indicates time of HS during day 6. (B, D) Bar graphs show mean $\pm$ s.e.m. percentage change in sleep during night 6 compared to night 5 from two combined experiments. (B) In the absence of the *hsp:Hcrt* transgene, HS during day 6 has no significant effect on the Sleep Night 6/Night 5 ratio, whether the animals are treated with prazosin or vehicle. (D) The reduced sleep induced by Hcrt overexpression is significantly diminished by prazosin. n indicates number of larvae. **, $p < 0.01$ and ns, not significant by one-way ANOVA.

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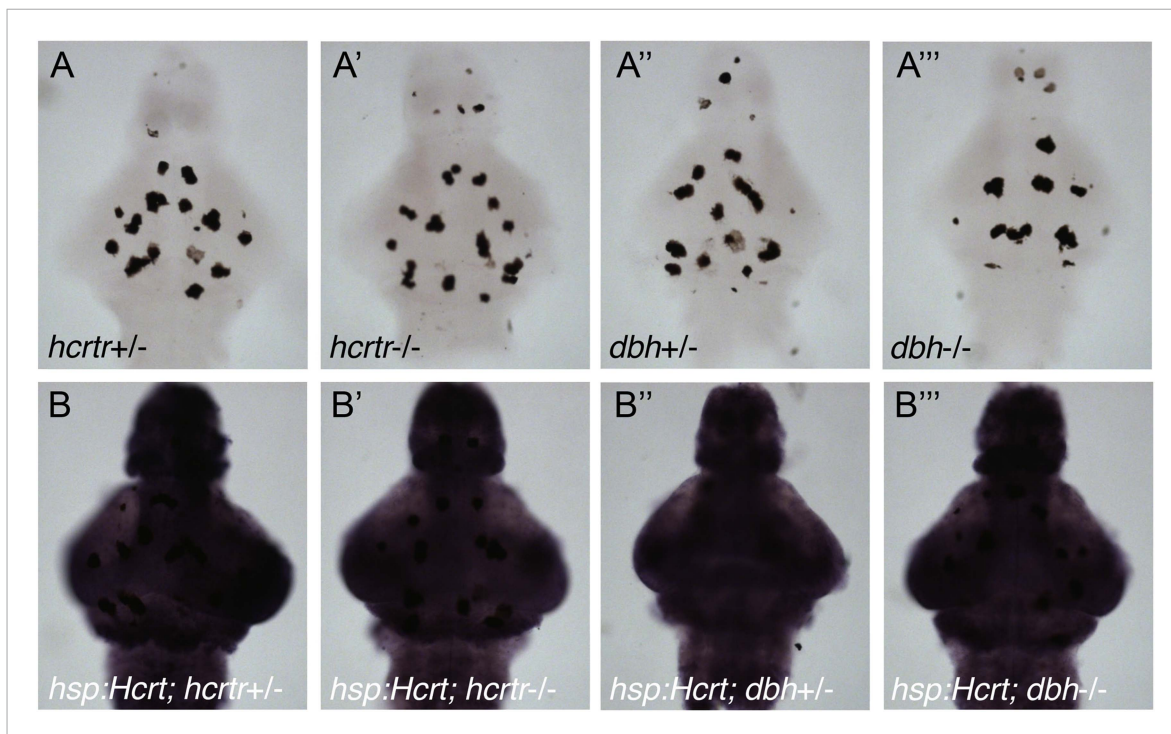


Figure 4—figure supplement 4. The heat shock promoter is not suppressed in *hcrtr* or *dbh* mutants. Representative images of brains from larvae that do not (A) or do (B) contain the *hsp:Hcrt* transgene, fixed 30 min after HS, and processed for ISH using a *hcrtr*-specific probe. *hcrtr* overexpression levels are similar for *hcrtr*+/- (B), *hcrtr*-/- (B'), *dbh*+/- (B'') and *dbh*-/- (B''') animals. Larvae were generated by crossing homozygous to heterozygous mutant animals, and brains were genotyped after imaging. Dark spots in (A–A''') are pigment cells. Endogenous *hcrtr* expression is not observed in (A–A''') because ISH development was stopped before endogenous *hcrtr* is detectable.

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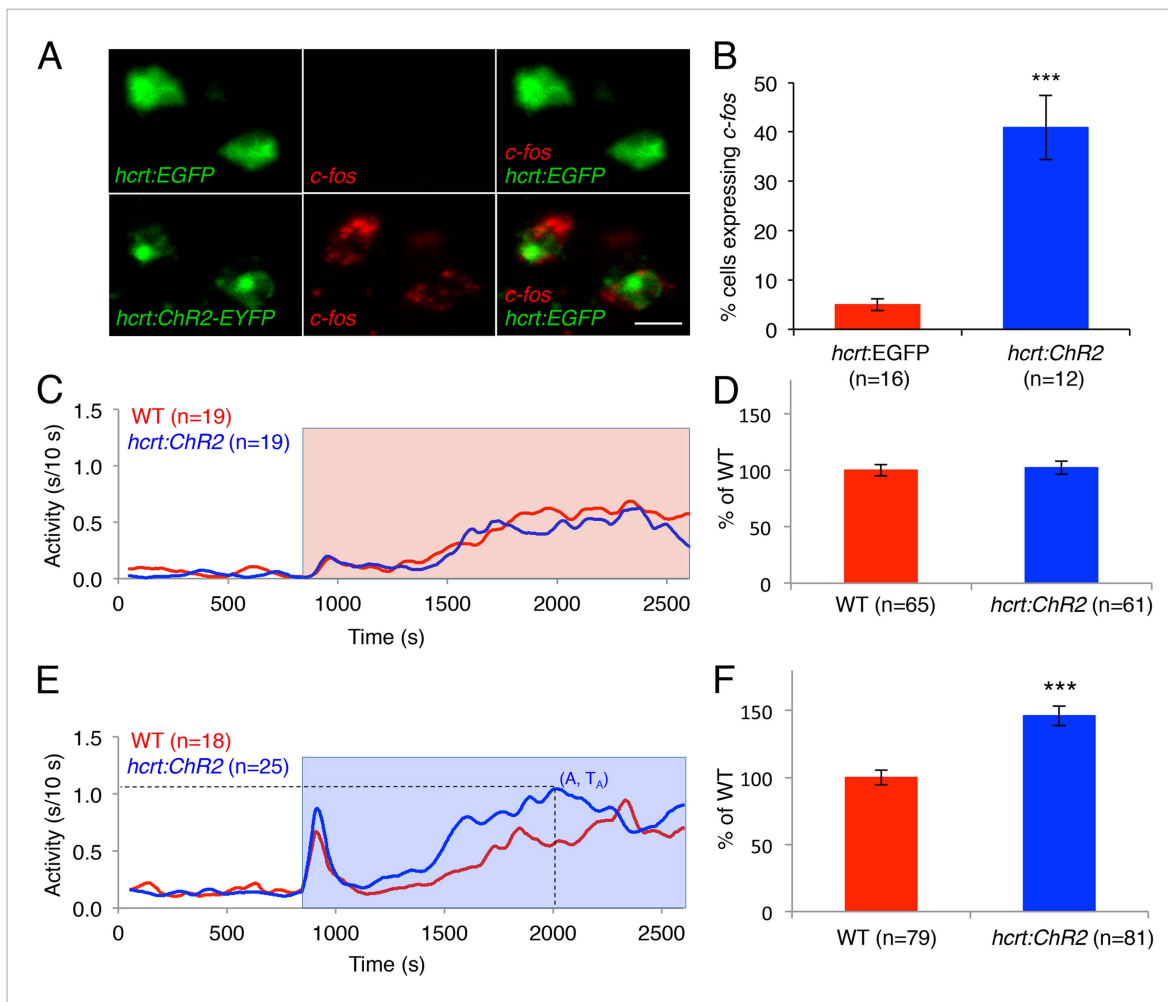


Figure 5. Optogenetic activation of Hcrt neurons increases locomotor activity. (A) Representative images of Hcrt neurons co-labeled with EYFP or EGFP (green) and *c-fos* (red). Scale bar = 10 μ m. (B) Mean $\pm$ s.e.m. percentage of EYFP- or EGFP-expressing neurons that also express *c-fos* in *Tg(hcrt:EGFP)* and *Tg(hcrt:ChR2-EYFP)* larvae. (C) Representative locomotor activity trace during red light exposure. (D) Average locomotor activity relative to WT siblings during red light exposure. Data is pooled from 3 experiments and is represented as mean $\pm$ s.e.m. (E) Representative locomotor activity trace during blue light exposure. (F) Average locomotor activity relative to WT siblings during blue light exposure. Data is pooled from 4 experiments and is represented as mean $\pm$ s.e.m. Red and blue boxes in (C) and (E) indicate periods of red and blue light illumination. 'A' is the maximum activity reached for larvae of a particular genotype and 'T_A' is the time taken to reach maximum activity. A. n indicates number of larvae. ***, $p < 0.0001$ by one-way ANOVA.

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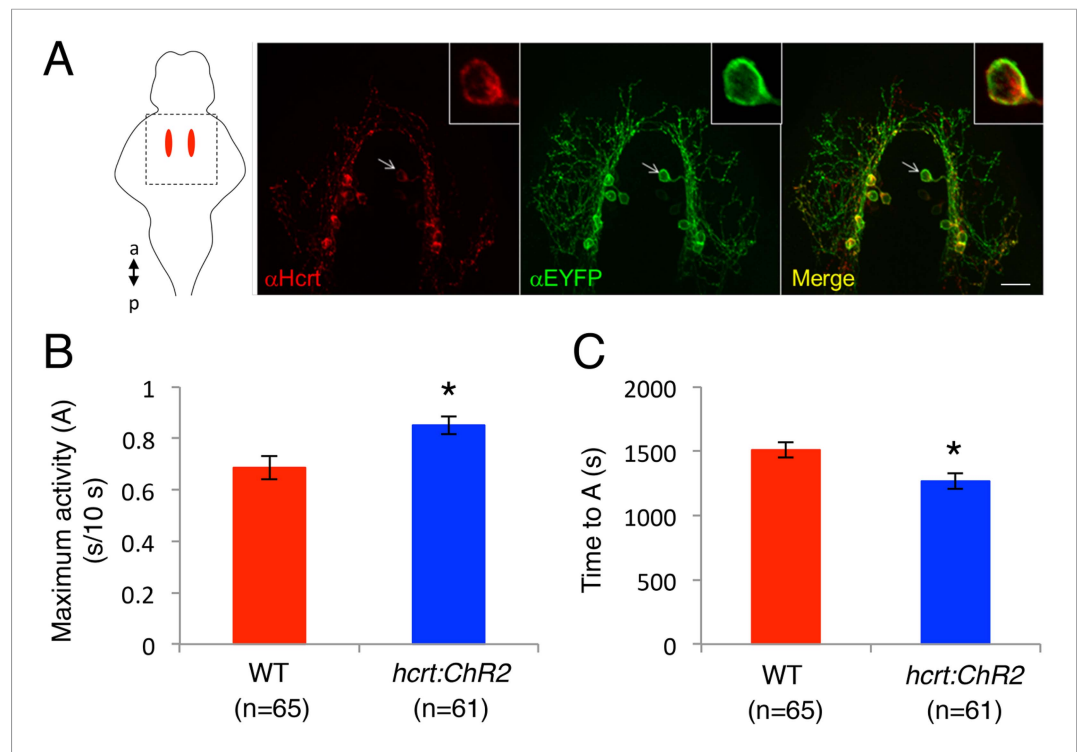


Figure 5—figure supplement 1. Specific expression of ChR2-EYFP in Hcrt neurons and temporal dynamics of *hcrt:ChR2-EYFP* induced locomotor activity. **(A)** Representative image from a *Tg(hcrt:ChR2-EYFP)* larval brain showing Hcrt (red) and EYFP (green) immunoreactivity, and merged images (yellow). Arrow indicates a neuron that is shown at higher magnification in the inset images. Schematic diagram indicates the brain region shown in the images. a = anterior, p = posterior. Scale bar = 20 μ m. **(B)** Activation of Hcrt neurons using ChR2 changes the dynamics of locomotor activity. *Tg(hcrt:ChR2-EYFP)* larvae reach a higher maximum level of locomotor activity **(B)**, and they do so faster **(C)**, than their non-transgenic siblings. 'A' is the maximum activity reached for larvae of a particular genotype and 'T_A' is the time taken to reach maximum activity, as diagrammed in **Figure 5**. Data represent mean $\pm$ s.e.m. n indicates number of larvae. *, p < 0.05 by one-way ANOVA.

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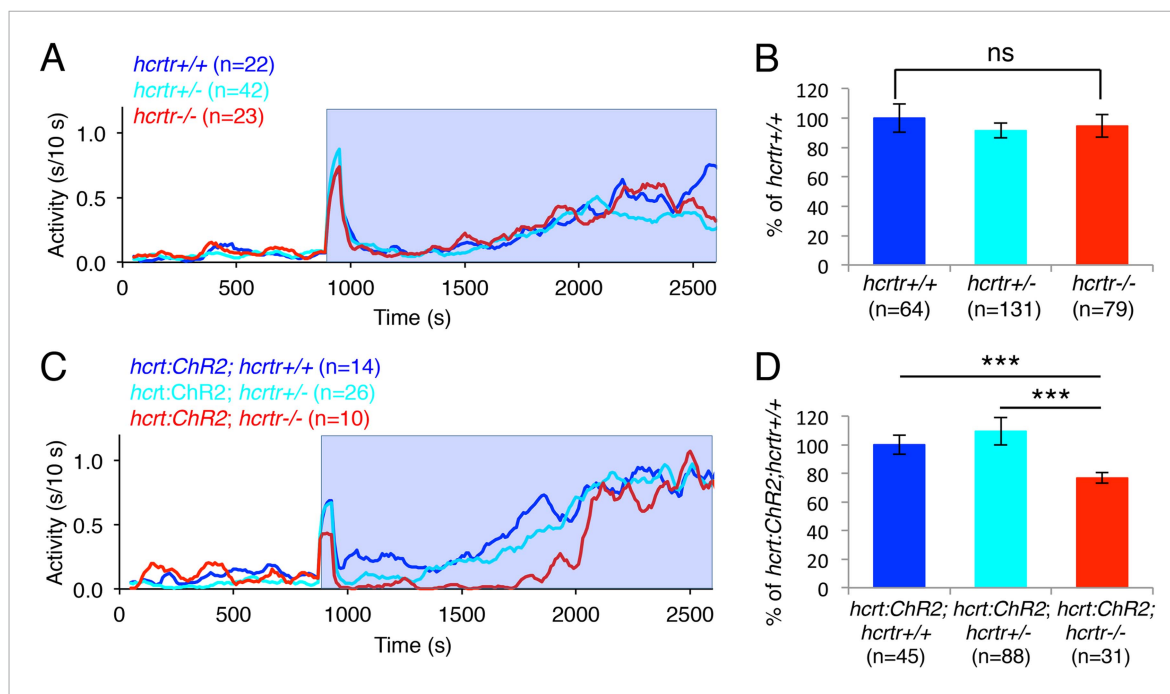


Figure 6. The *hcrtr* is required for Hcrt neuron-induced increased locomotor activity. (**A**, **C**) Representative locomotor activity traces for *hcrtr*^{-/-} and sibling controls without (**A**) and with (**C**) the *hcrtr:ChR2-EYFP* transgene during blue light exposure. (**B**, **D**) Average locomotor activity relative to sibling controls. Data is pooled from 3 experiments in both cases. Data is represented as mean $\pm$ s.e.m. and is plotted as percentage of *hcrtr*^{+/+} (**B**) or *hcrtr:ChR2-EYFP; hcrtr*^{+/+} (**D**). n indicates number of larvae. ***, $p < 0.0001$ and ns, not significant by one-way ANOVA followed by Tukey's test to correct for multiple comparisons. DOI: 10.7554/eLife.07000.018

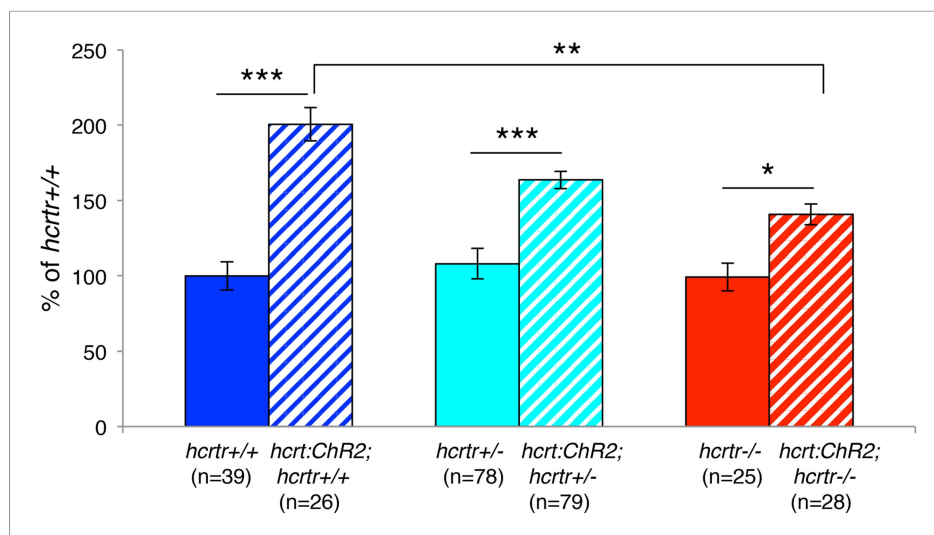


Figure 6—figure supplement 1. Hcrt signaling is required for Hcrt neuron-induced increased locomotor activity. In this experiment, larvae of all six genotypes were tested in the same behavioral plates. *Tg(hcrt:ChR2-EYFP);hcrtr+/+* larvae (blue stripes) showed a significant increase in locomotor activity compared to *hcrtr+/+* larvae (blue). A similar increase was observed for *Tg(hcrt:ChR2-EYFP);hcrtr+/-* larvae (cyan stripes) compared to *hcrtr+/-* larvae (cyan), although the difference was smaller than for *hcrtr+/+* larvae, suggesting a dosage effect. *Tg(hcrt:ChR2-EYFP);hcrtr-/-* larvae (red stripes) were also more active than *hcrtr-/-* larvae (red), although the difference was much smaller than for *Tg(hcrt:ChR2-EYFP);hcrtr+/+* larvae (blue stripes) compared to *hcrtr+/+* larvae (blue). *Tg(hcrt:ChR2-EYFP);hcrtr+/+* larvae (blue stripes) were also significantly more active than *Tg(hcrt:ChR2-EYFP);hcrtr-/-* larvae (red stripes). These results suggest that most, but not all, of the effect of stimulating Hcrt neurons on locomotor activity is due to Hcrt signaling. Data is pooled from 3 experiments. Data is represented as mean $\pm$ s.e.m. and is plotted as percentage of *hcrtr+/+*. n indicates number of larvae. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ by Steel–Dwass test and correction for multiple comparisons.

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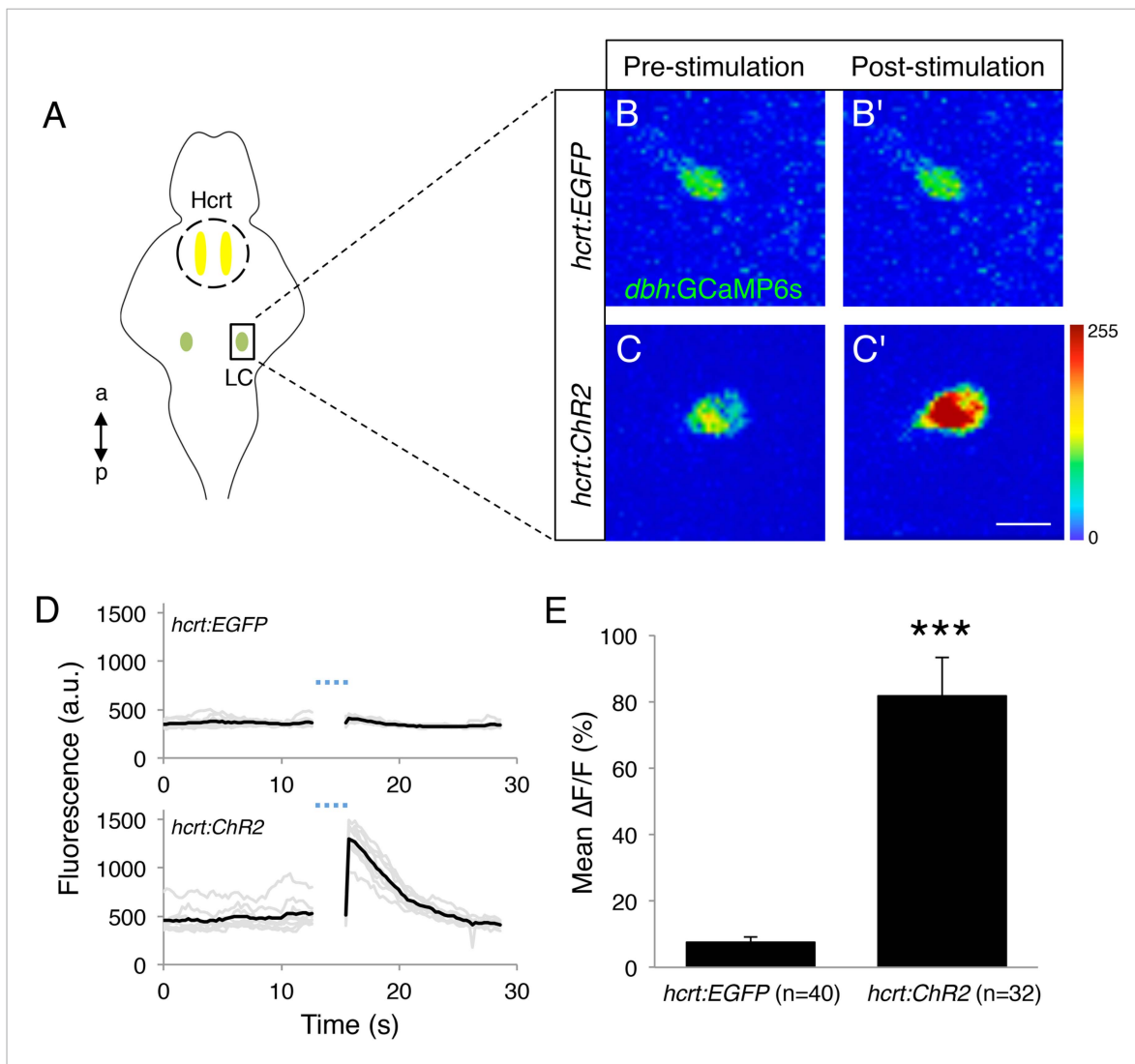


Figure 7. Optogenetic stimulation of Hcrt neurons activates LC neurons. **(A)** Schematic representation of areas stimulated (Hcrt, yellow) and imaged (LC, green). a = anterior, p = posterior. **(B, C)** Representative images of a LC cell expressing GCaMP6s in a *Tg(hcrt:EGFP)* (B) or *Tg(hcrt:ChR2-EYFP)* (C) larva before (B, C) and immediately after (B', C') stimulation of Hcrt neuron region. **(D)** GCaMP6s fluorescence intensity for a representative LC neuron in a *Tg(hcrt:EGFP)* (top) and a *Tg(hcrt:ChR2-EYFP)* (bottom) larva. Single-trial (gray) and average (black) responses are shown. **(E)** Mean $\pm$ s.e.m. $\Delta F/F$ (%) values averaged for all trials. n indicates number of trials for 5 *Tg(hcrt:EGFP)* and 4 *Tg(hcrt:ChR2-EYFP)* larvae. ***, $p < 0.001$ by one-way ANOVA. See **Videos 1 and 2** for examples of GCaMP6s imaging.

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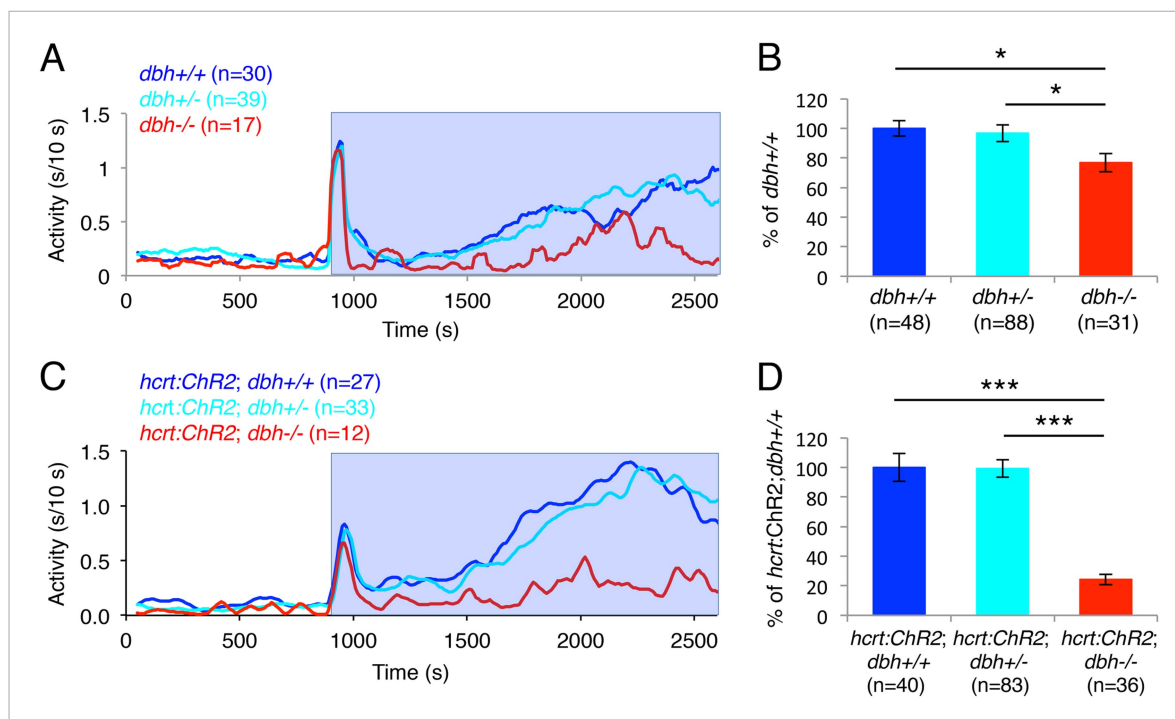


Figure 8. *dbh* is required for Hcrt neuron-induced increased locomotor activity. **(A, C)** Representative locomotor activity traces for *dbh*^{-/-} and sibling control larvae without **(A)** and with **(C)** the *hcr:ChR2-EYFP* transgene during blue light exposure. **(B, D)** Average locomotor activity relative to sibling controls. Data is pooled from 2 **(B)** or 3 **(D)** experiments. Data is represented as mean $\pm$ s.e.m. and is plotted as percentage of *dbh*^{+/+} **(B)** or *hcr:ChR2-EYFP; dbh*^{+/+} **(D)**. n indicates number of larvae. *, $p < 0.05$ and ***, $p < 0.0001$ by one-way ANOVA followed by Tukey's test to correct for multiple comparisons.

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