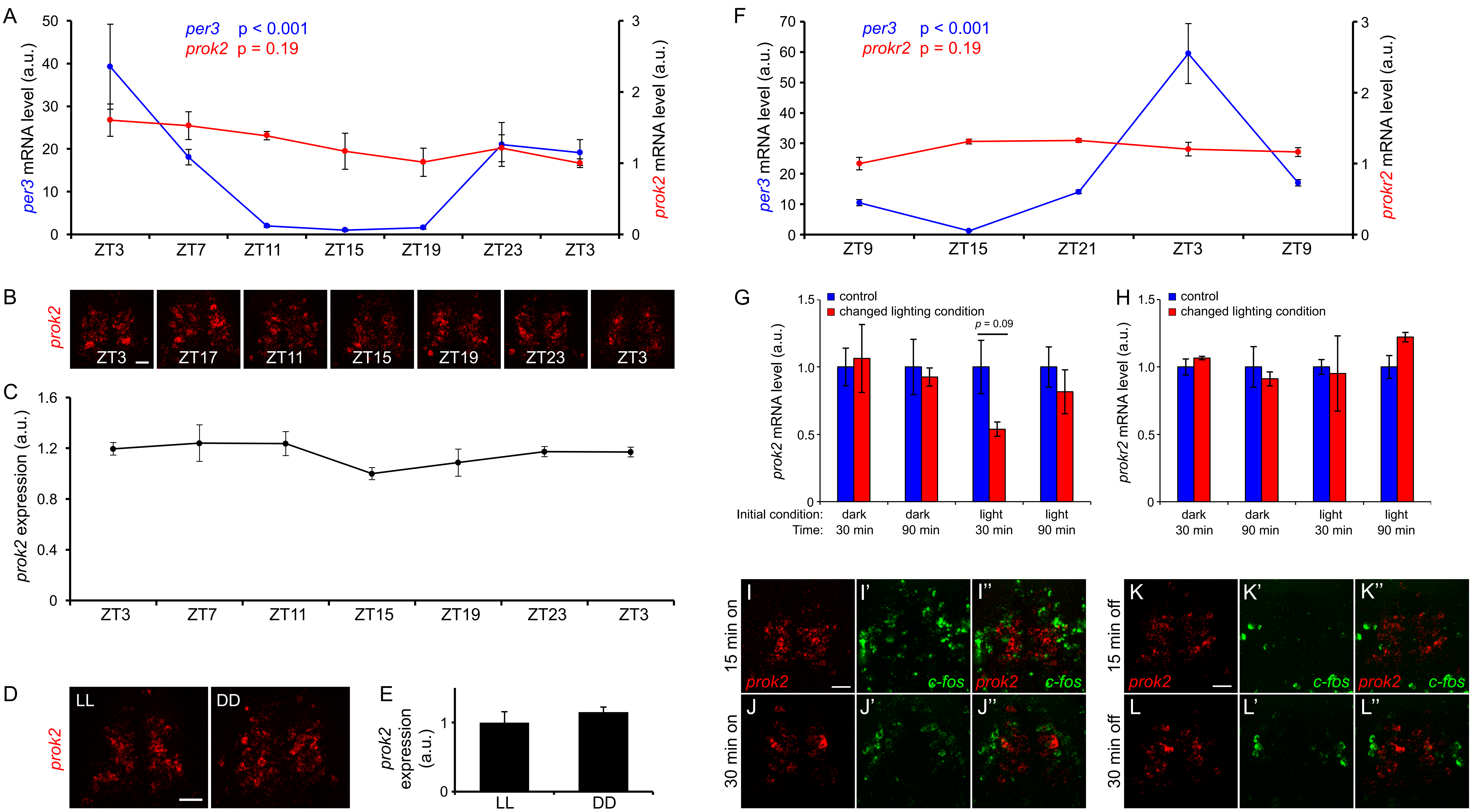
Figure S1



A
per3 p < 0.001
prok2 p = 0.19
per3 mRNA level (a.u.)
prok2 mRNA level (a.u.)
ZT3 ZT7 ZT11 ZT15 ZT19 ZT23 ZT3
B
prok2
ZT3 ZT17 ZT11 ZT15 ZT19 ZT23 ZT3
C
prok2 expression (a.u.)
ZT3 ZT7 ZT11 ZT15 ZT19 ZT23 ZT3
D
prok2
LL DD
E
prok2 expression (a.u.)
LL DD
F
per3 p < 0.001
prokr2 p = 0.19
per3 mRNA level (a.u.)
prokr2 mRNA level (a.u.)
ZT9 ZT15 ZT21 ZT3 ZT9
G
control
changed lighting condition
p = 0.09
prok2 mRNA level (a.u.)
Initial condition: dark dark light light
Time: 30 min 90 min 30 min 90 min
H
control
changed lighting condition
prokr2 mRNA level (a.u.)
dark 30 min dark 90 min light 30 min light 90 min
15 min on
30 min on
I I' I''
J J' J''
prok2 c-fos prok2 c-fos
15 min off
30 min off
K K' K''
L L' L''
prok2 c-fos prok2 c-fos

Figure S2

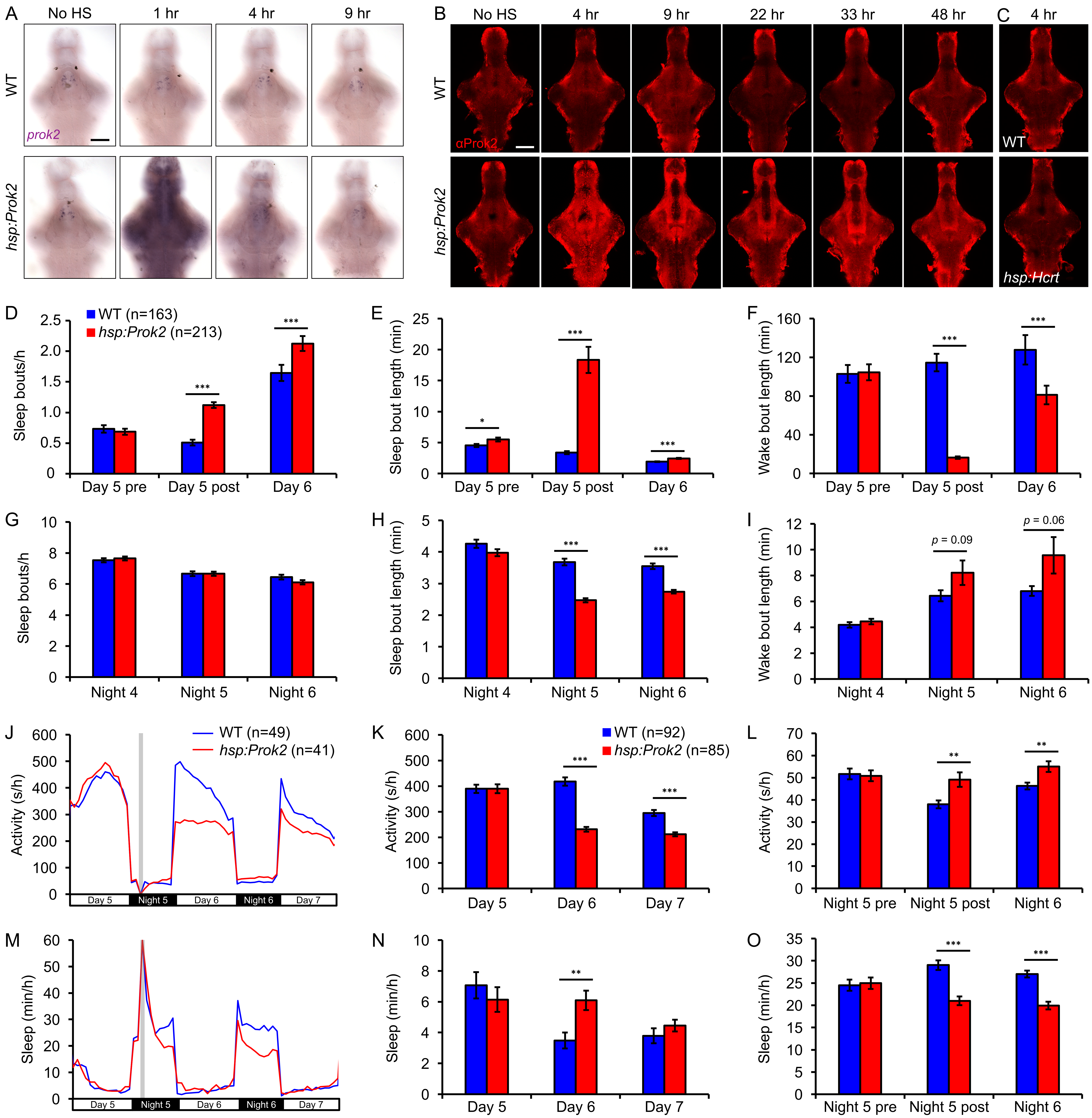
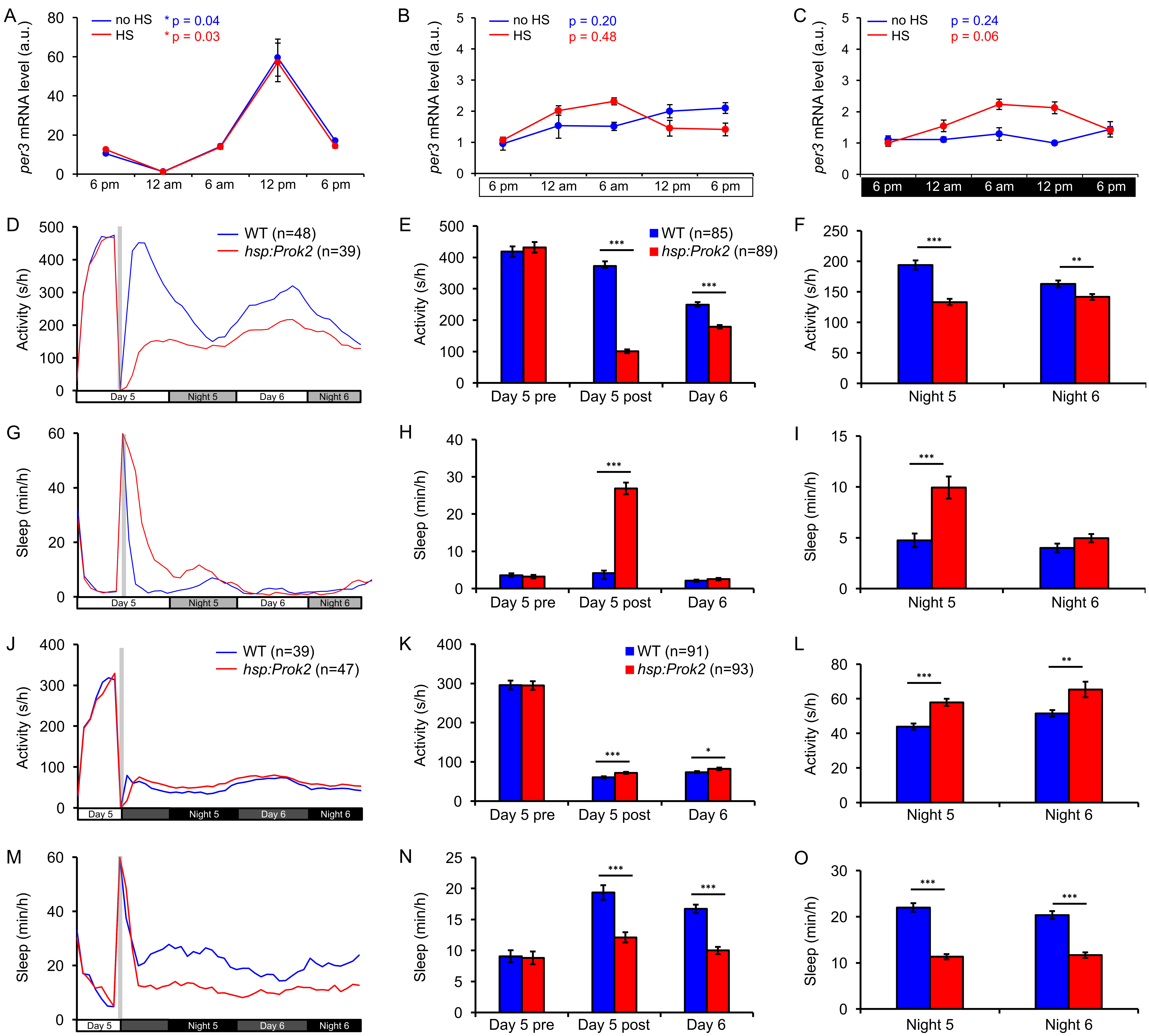
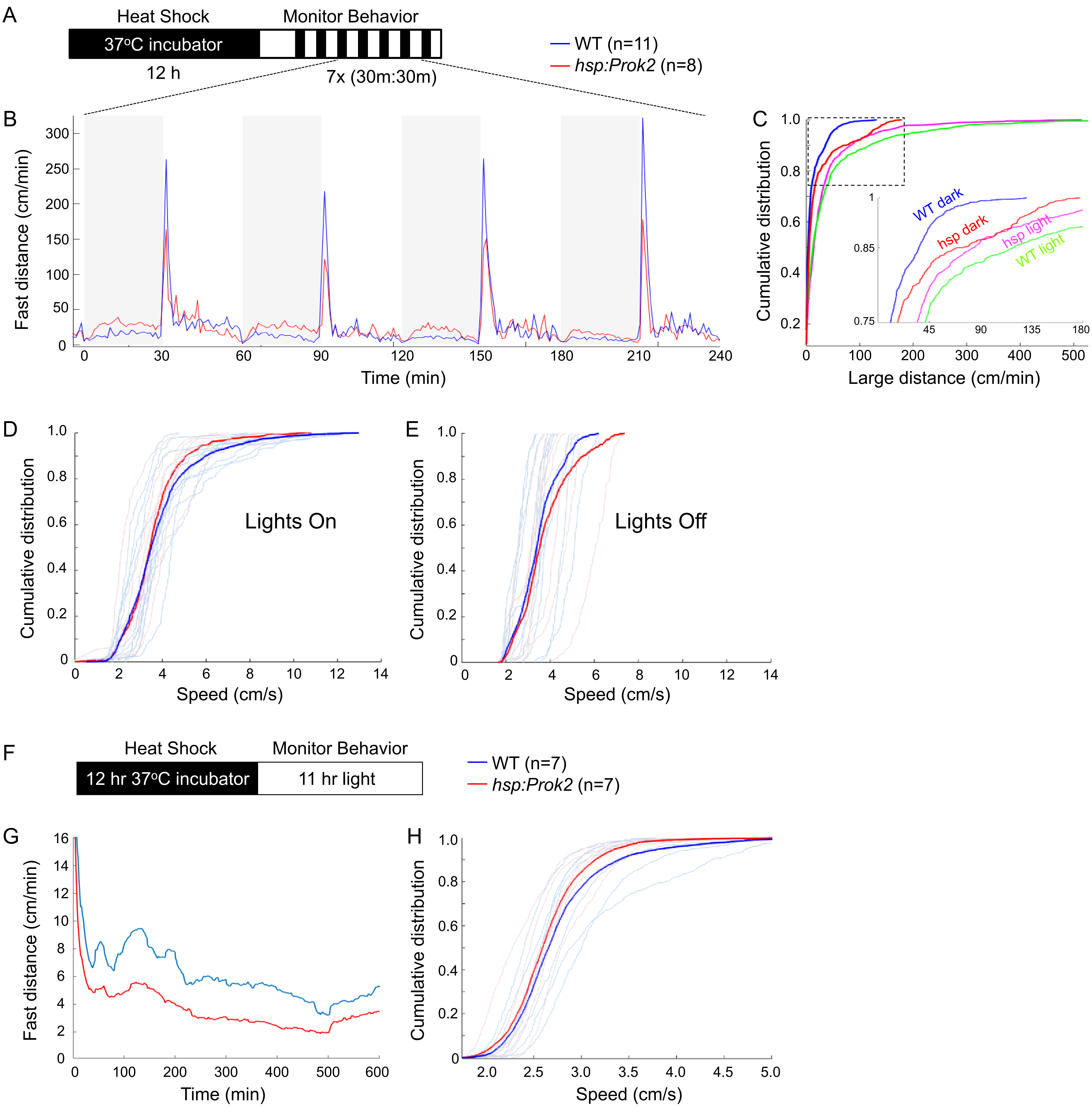

A
No HS
1 hr
4 hr
9 hr
WT
hsp:Prok2
prok2
B
No HS
4 hr
9 hr
22 hr
33 hr
48 hr
αProk2
C
4 hr
WT
hsp:Hcrt
D
WT (n=163)
hsp:Prok2 (n=213)
Sleep bouts/h
Day 5 pre
Day 5 post
Day 6
E
Sleep bout length (min)
F
Wake bout length (min)
G
Sleep bouts/h
Night 4
Night 5
Night 6
H
Sleep bout length (min)
I
Wake bout length (min)
p = 0.09
p = 0.06
J
WT (n=49)
hsp:Prok2 (n=41)
Activity (s/h)
Day 5
Night 5
Day 6
Night 6
Day 7
K
WT (n=92)
hsp:Prok2 (n=85)
Activity (s/h)
L
Activity (s/h)
Night 5 pre
Night 5 post
Night 6
M
Sleep (min/h)
N
Sleep (min/h)
O
Sleep (min/h)

Figure S3



A
per3 mRNA level (a.u.)
no HS
HS
* p = 0.04
* p = 0.03
6 pm
12 am
6 am
12 pm
6 pm
B
p = 0.20
p = 0.48
C
p = 0.24
p = 0.06
D
Activity (s/h)
WT (n=48)
hsp:Prok2 (n=39)
Day 5
Night 5
Day 6
Night 6
E
WT (n=85)
hsp:Prok2 (n=89)
Day 5 pre
Day 5 post
Day 6
F
Night 5
Night 6
G
Sleep (min/h)
H
I
J
WT (n=39)
hsp:Prok2 (n=47)
K
WT (n=91)
hsp:Prok2 (n=93)
L
M
N
O

Figure S4



A
Heat Shock
Monitor Behavior
37°C incubator
12 h
7x (30m:30m)
WT (n=11)
hsp:Prok2 (n=8)
B
Fast distance (cm/min)
Time (min)
C
Cumulative distribution
Large distance (cm/min)
WT dark
hsp dark
hsp light
WT light
D
Cumulative distribution
Lights On
Speed (cm/s)
E
Cumulative distribution
Lights Off
Speed (cm/s)
F
Heat Shock
Monitor Behavior
12 hr 37°C incubator
11 hr light
WT (n=7)
hsp:Prok2 (n=7)
G
Fast distance (cm/min)
Time (min)
H
Cumulative distribution
Speed (cm/s)

Figure S5

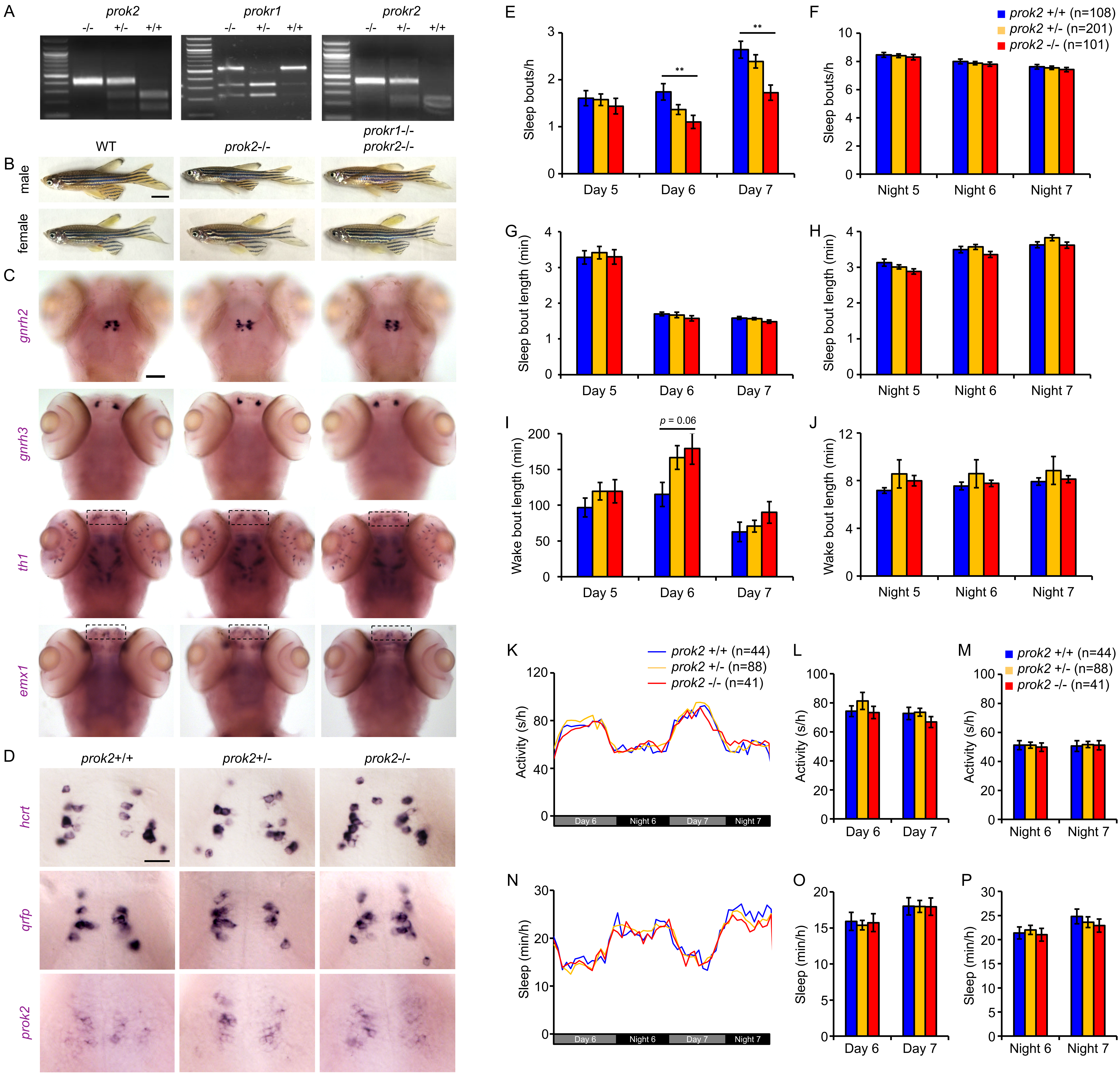
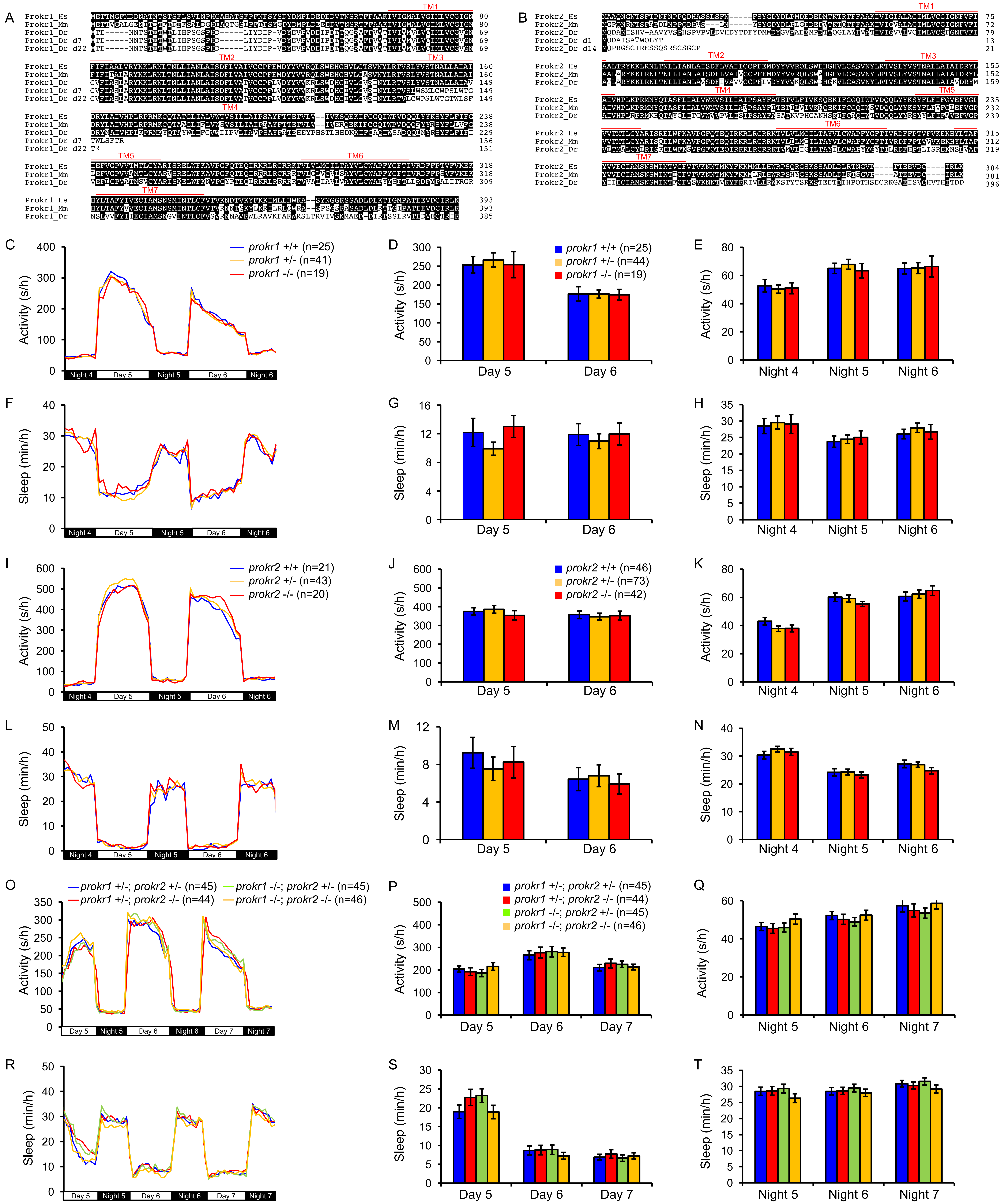
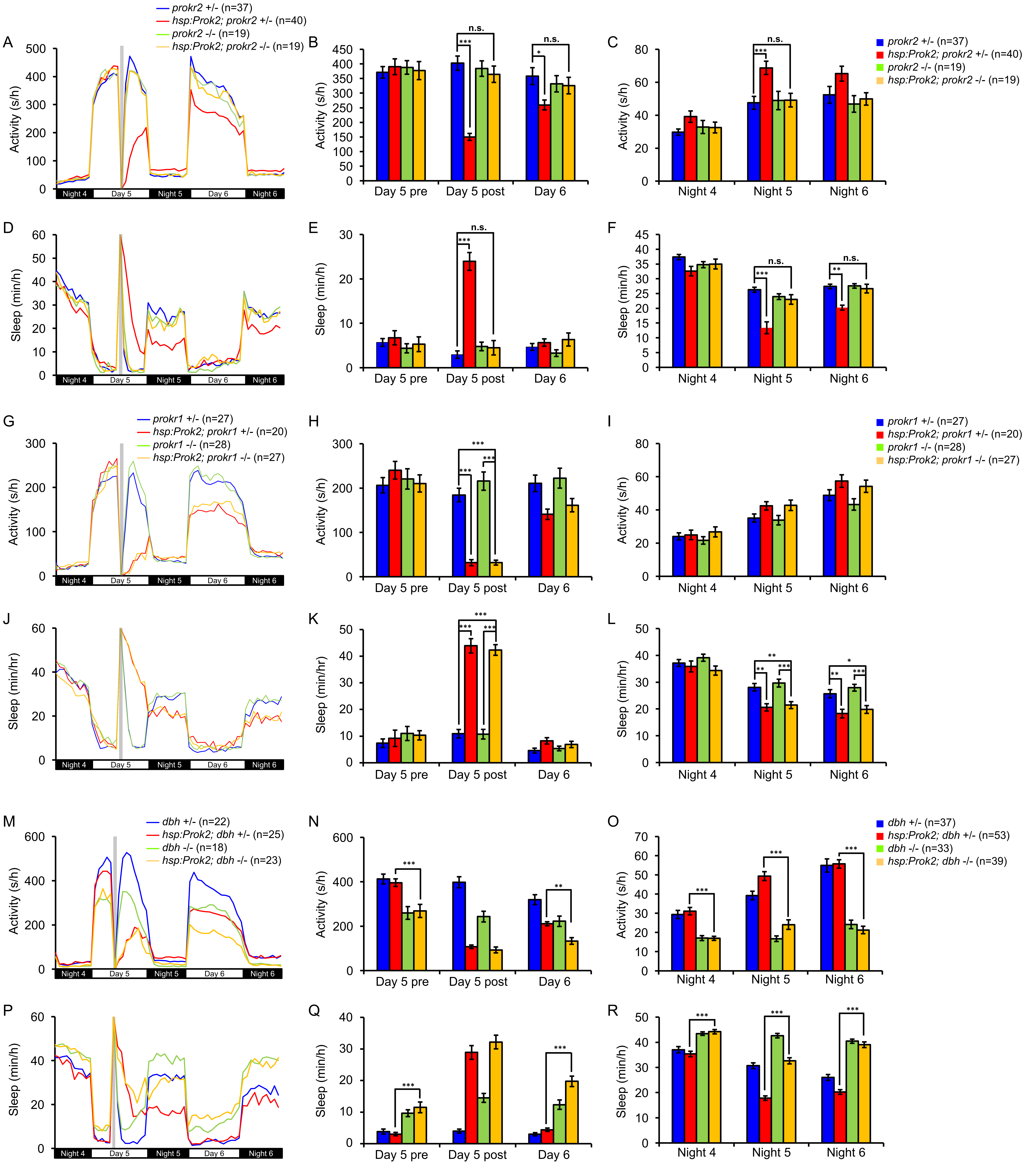

A
prok2
prokr1
prokr2
-/-
+/-
+/+
B
WT
prok2-/-
prokr1-/-
prokr2-/-
male
female
C
gnrh2
gnrh3
th1
emx1
D
prok2+/+
prok2+/-
prok2-/-
hcrt
qrfp
prok2
E
Sleep bouts/h
Day 5
Day 6
Day 7
**
F
prok2 +/+ (n=108)
prok2 +/- (n=201)
prok2 -/- (n=101)
Night 5
Night 6
Night 7
G
Sleep bout length (min)
H
I
Wake bout length (min)
p = 0.06
J
K
prok2 +/+ (n=44)
prok2 +/- (n=88)
prok2 -/- (n=41)
Activity (s/h)
Day 6
Night 6
Day 7
Night 7
L
M
N
Sleep (min/h)
O
P

Figure S6



A
B
TM1
TM2
TM3
TM4
TM5
TM6
TM7
Prokr1_Hs
Prokr1_Mm
Prokr1_Dr
Prokr1_Dr d7
Prokr1_Dr d22
Prokr2_Hs
Prokr2_Mm
Prokr2_Dr
Prokr2_Dr d1
Prokr2_Dr d14
C
prokr1 +/+ (n=25)
prokr1 +/- (n=41)
prokr1 -/- (n=19)
Activity (s/h)
Night 4
Day 5
Night 5
Day 6
Night 6
D
prokr1 +/+ (n=25)
prokr1 +/- (n=44)
prokr1 -/- (n=19)
Activity (s/h)
Day 5
Day 6
E
Activity (s/h)
Night 4
Night 5
Night 6
F
Sleep (min/h)
G
Sleep (min/h)
H
Sleep (min/h)
I
prokr2 +/+ (n=21)
prokr2 +/- (n=43)
prokr2 -/- (n=20)
Activity (s/h)
J
prokr2 +/+ (n=46)
prokr2 +/- (n=73)
prokr2 -/- (n=42)
Activity (s/h)
K
Activity (s/h)
L
Sleep (min/h)
M
Sleep (min/h)
N
Sleep (min/h)
O
prokr1 +/-; prokr2 +/- (n=45)
prokr1 +/-; prokr2 -/- (n=44)
prokr1 -/-; prokr2 +/- (n=45)
prokr1 -/-; prokr2 -/- (n=46)
Activity (s/h)
Day 5
Night 5
Day 6
Night 6
Day 7
Night 7
P
prokr1 +/-; prokr2 +/- (n=45)
prokr1 +/-; prokr2 -/- (n=44)
prokr1 -/-; prokr2 +/- (n=45)
prokr1 -/-; prokr2 -/- (n=46)
Activity (s/h)
Day 5
Day 6
Day 7
Q
Activity (s/h)
Night 5
Night 6
Night 7
R
Sleep (min/h)
S
Sleep (min/h)
T
Sleep (min/h)

Figure S7



A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
prokr2 +/- (n=37)
hsp:Prok2; prokr2 +/- (n=40)
prokr2 -/- (n=19)
hsp:Prok2; prokr2 -/- (n=19)
prokr1 +/- (n=27)
hsp:Prok2; prokr1 +/- (n=20)
prokr1 -/- (n=28)
hsp:Prok2; prokr1 -/- (n=27)
dbh +/- (n=22)
hsp:Prok2; dbh +/- (n=25)
dbh -/- (n=18)
hsp:Prok2; dbh -/- (n=23)
dbh +/- (n=37)
hsp:Prok2; dbh +/- (n=53)
dbh -/- (n=33)
hsp:Prok2; dbh -/- (n=39)
Activity (s/h)
Sleep (min/h)
Sleep (min/hr)
Night 4
Day 5
Night 5
Day 6
Night 6
Day 5 pre
Day 5 post
n.s.

Figure S8

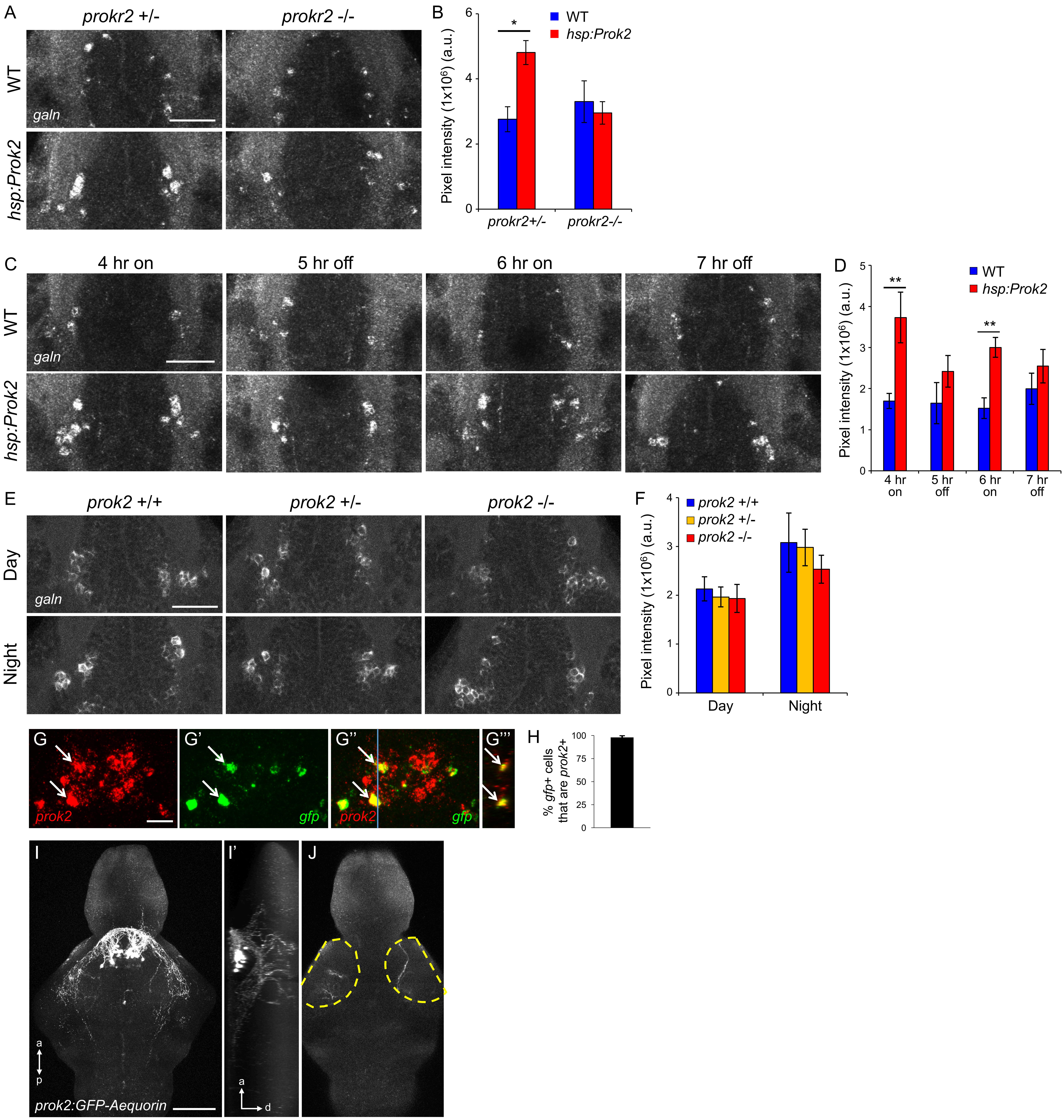

A
prokr2 +/-
prokr2 -/-
WT
hsp:Prok2
galn
B
WT
hsp:Prok2
Pixel intensity (1x10^6) (a.u.)
prokr2+/-
prokr2-/-
C
4 hr on
5 hr off
6 hr on
7 hr off
WT
hsp:Prok2
galn
D
WT
hsp:Prok2
Pixel intensity (1x10^6) (a.u.)
4 hr on
5 hr off
6 hr on
7 hr off
E
prok2 +/+
prok2 +/-
prok2 -/-
Day
Night
galn
F
prok2 +/+
prok2 +/-
prok2 -/-
Pixel intensity (1x10^6) (a.u.)
Day
Night
G
G'
G''
G'''
prok2
gfp
H
% gfp+ cells that are prok2+
I
I'
J
a
p
d
prok2:GFP-Aequorin

Table S1: Primers and genome editing target sequences	
Genotyping Primers	
Primer Name	**Primer Sequence**
hsp:Prok2 genotype F	CGGGCCACCATGACAT
hsp:Prok2 genotype R	GGTTTGTCCAAACTCATCAATGT
prok2 mutant genotype F	CAAGTGGACACACCGAACAC
prok2 mutant genotype R	ATCCTGGAATGGAAATGGTG
prokr1 mutant genotype F	GGCGTTGGTAATTGCGTATT
prokr1 mutant genotype R	TATCAGCCACCAGCACTCTG
prokr2 mutant genotype F	TGAGCGTAATGCTAATGGTCT
prokr2 mutant genotype R	CCAGAGTGGCGATAAACACA
galn mutant genotype F	ATGTACTGTCCTCATGGCAAAG
galn mutant genotype R	AAATGTAGACCTGAGAGCAGC
qPCR Primers	
Primer Name	**Primer Sequence**
prok2 qPCR F	GGGGCATGTGAGAAGGACTCT
prok2 qPCR R	TCTTCTCCCTCCTGACCCATT
prokr2 qPCR F	GTGTCCACCAACGCCTTACT
prokr2 qPCR R	CTCCTGTGATCAAACAGTACGC
per3 qPCR F	CTCCAGCTTTCACAGCACTCA
per3 qPCR R	ACGCTTCTTCATCTCCTGCAC
actin qPCR F	TCCTCCCTGGAGAAGAGCTATG
actin qPCR R	TCCATACCCAGGAAGGAAGG
Primers for Riboprobe Synthesis	
Primer Name	**Primer Sequence**
prokr1 riboprobe F	TGACTCGCAGTCACACAGTTC
prokr1 riboprobe T7 R	GAATTGTAATACGACTCACTATAGGGTCTTCCAAAGTATGGGTCGAA
prokr2 riboprobe F	GCACAGAGAATGAGCGTCTG
prokr2 riboprobe T7 R	GAATTGTAATACGACTCACTATAGGGTCACTGAGGCTGAGGGTATAAA
grp riboprobe F	CTATGTGCCTGGTGTGGAGA
grp riboprobe T7 R	GAATTGTAATACGACTCACTATAGGGGCAGAAAGCCCAACAAGTTC
tgfα riboprobe F	CGCGTGCCTTCATCTTTATT
tgfα riboprobe T7 R	GAATTGTAATACGACTCACTATAGGGTCCCACTGCCCATATTGAAC
TALEN and CRISPR/Cas9 DNA Target Sequences	
Target Name	**Sequence**
prok2 TALEN L	TGGCATGTGTTGTGCAGT
prok2 TALEN R	TGCACATTCGGAGACT
prokr1 TALEN L	TACTTGAGGACTGTGTC
prokr1 TALEN R	TGGCCAGCAGAGCATT
prokr2 TALEN L	TCTCACAGAAACAGCCAT
prokr2 TALEN R	TACAGCTGCCACGTGGCT
galn sgRNA	CGGACTCACGAGGACCGAGGA

Supplemental Figure Legends

Figure S1. *prok2* and *prokr2* expression levels do not oscillate in circadian manner, *prok2* expression level is not affected by light, and changes in light do not activate *prok2*-expressing neurons in larval zebrafish. Related to Figure 1. (A, F) qRT-PCR analysis of *prok2* and *per3* (A), and *prokr2* and *per3* (F), mRNA is shown. Larvae were raised in 14:10 hour LD conditions and collected at the indicated times beginning at 6 dpf. Mean ± SEM for triplicate biological samples normalized to *actin* at each time point is shown. *prok2* and *prokr2* expression does not change significantly over 24 hours, while *per3* expression oscillates with a 24-hour period. P values were calculated using one-way ANOVA. (B) 53 µm thick confocal projections showing *prok2* FISH in larval zebrafish brains fixed at the indicated times. (C) Quantification of total *prok2* fluorescence pixel intensity is shown. *prok2* expression does not oscillate (peak:trough ratio=1.24, p=0.40 by one-way ANOVA). Mean ± SEM for five brains at each time point is shown. a.u. = arbitrary units. (D) A 53 µm thick confocal projection showing *prok2* FISH in brains of larvae raised in LL or DD and fixed at 6 dpf. (E) Mean ± SEM *prok2* FISH fluorescence intensity for 5 brains in each condition is shown, normalized to the LL condition. There is no significant difference in *prok2* mRNA level between the two conditions (p=0.40 by two-tailed Student's *t* test). (G, H) Larvae were raised until 6 dpf in either DD or LL, then exposed to light or dark, respectively. Samples were fixed at the indicated times after the change in lighting condition. Control larvae were maintained in the original lighting condition. qRT-PCR was performed to measure the level of *prok2* (G) or *prokr2* (H) mRNA. Mean ± SEM for triplicate biological samples normalized to *actin* at each time point is shown. Statistical significance was assessed by two-tailed Student's *t* test. (I-L) *prok2*-expressing neurons do not express *c-fos* in response to changes in lighting conditions. Larvae raised in DD (I, J) or LL (K, L) until 5 dpf were transferred to light (I, J) or dark (K, L) conditions, and fixed 15 or 30 minutes later. Double FISH using *prok2*- and *c-fos*-specific probes showed that *c-fos* is not expressed in *prok2*-expressing neurons in either case. Scale bars: 10 µm.

Figure S2. Dynamics of Prok2 overexpression, effects of induction of Prok2 overexpression during the day on sleep architecture, and induction of Prok2 overexpression at night produces a phenotype similar to that of daytime induction. Related to Figure 2. (A-B) Time course of *prok2* mRNA and protein overexpression. Representative images of ISH using a *prok2*-specific probe (A) or IHC using a Prok2-specific antibody (B) in WT and *Tg(hsp:Prok2)* larvae. Larvae were heat shocked from 1-2 p.m. at 5 dpf and fixed at the indicated times after heat shock. Ectopic *prok2* mRNA is detected for at least 4 hours after heat shock. Ectopic Prok2 protein can be detected for at least 48 hours after heat shock. (C) Representative images of IHC using a Prok2-specific antibody on 5 dpf WT and *Tg(hsp:Hcrt)* larvae fixed 4 hours after heat shock. Overexpressed Hcrt protein (Prober et al., 2006) is not detected by the Prok2-specific antibody. Brains were genotyped by PCR after imaging. No HS, no heat shock. Scale bars: 100 µm. (D-I) Following a daytime heat shock, increased daytime sleep due to Prok2 overexpression results from an increase in the number (D) and length (E) of sleep bouts, with a corresponding decrease in the length of wake bouts (F). Decreased sleep at night due to Prok2 overexpression results from a decrease in the length of sleep bouts (H). (J-O) Heat shock (gray bar in (J, M)) at night results in more activity at night (J, L) and less activity during the day (J, K) for *Tg(hsp:Prok2)* larvae compared to their WT siblings. Prok2 overexpression also results in less sleep at night (M, O) and more sleep during the day (M, N). These phenotypes were observed for up to 36 hours following heat shock. Note that (K, L, N, O) exclude the first two hours after heat shock to allow larvae to recover from the heat shock. Both genotypes slept more during this recovery period. Data from one representative experiment (J, M), two experiments combined (K, L, N, O) and five experiments combined (D-I) are shown. (D-I, K, L, N, O) show mean ± SEM. n = number of larvae. *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student's *t* test.

Figure S3. Heat shock does not affect circadian rhythms, and the Prok2 overexpression phenotype depends on lighting conditions and not on circadian rhythms. Related to Figures 3 and 4. (A-C) qRT-PCR analysis of *per3* mRNA levels in larvae raised in LD (A), LL (B) or DD (C). *per3* levels oscillate in LD but not in LL or DD. At 6 dpf, larvae were (red) or were not (blue) heat shocked at 3 p.m. *per3* levels were not significantly affected by heat shock in any of the lighting conditions. Note the difference in y-axis scale for LD compared to LL and LD. Mean ± SEM for triplicate biological samples normalized to *actin* at each time point is shown. (D-I) *Tg(hsp:Prok2)* larvae entrained in 14:10 hour LD conditions for 5 days and tested in LL show less locomotor activity (D-F) and more sleep (G-I) after heat shock (gray bar in (D, G)) compared to their WT siblings during both subjective day and subjective night. (J-O) *Tg(hsp:Prok2)* larvae entrained in 14:10 hour LD conditions for 5 days and tested in DD show more locomotor activity (J-L) and less sleep (M-O) after heat shock (gray bar in (J, M)) compared to their WT siblings during both subjective day and subjective night. Data from one representative experiment (D, G, J, M) and two experiments combined (E, F, H, I, K, L, N, O) are shown. Bar graphs show mean ± SEM. n = number of larvae. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA (A-C) or two-tailed Student's *t* test (E, F, H, I, K, L, N, O).

Figure S4. Prok2 overexpression in adults increases locomotor activity in dark and suppresses locomotor activity in light. Related to Figures 2 and 4. (A) Experimental protocol for (B-E). After a 12-hour heat shock at 37^{o}C in the dark, *Tg(hsp:Prok2)* adult fish and their WT siblings were monitored during alternating 30 minute periods of light and dark. (B) Prok2 overexpression increased the distance travelled at fast speeds in the dark (gray shading), and also decreased the distance travelled in the light, especially in the initial minutes after dark to light transitions. (C) Cumulative distribution plot (boxed region expanded in inset) of all distances travelled shows that both WT and *Tg(hsp:Prok2)* adults were more active in the light (WT light, green; hsp light, magenta) than in the dark (WT dark, blue; hsp dark, red). In the dark, *Tg(hsp:Prok2)* fish swam larger distances than WT, while in the light, *Tg(hsp:Prok2)* fish swam smaller distances than WT, similar to larval zebrafish. (D) Cumulative distribution of all swimming speeds in the light by *Tg(hsp:Prok2)* adult fish (light red, single fish; dark red, average of all fish) and their WT siblings (light blue, single fish; dark blue, average of all fish). *Tg(hsp:Prok2)* fish exhibited fewer bouts of high speed than their WT siblings (p=0.002, Kolmogorov–Smirnov test). (E) Cumulative distribution of all swimming speeds in the dark. *Tg(hsp:Prok2)* adult fish exhibited more high speed bouts than their WT siblings ($p=3.5\times10^{-5}$, Kolmogorov–Smirnov test). (F) Experimental protocol for (G-H). After a 12-hour heat shock at 37^{o}C in the dark, *Tg(hsp:Prok2)* adult fish and their WT siblings were monitored during 11 hours of light. (G) Prok2 overexpression reduced the distance traveled at fast speeds in light. (H). Cumulative distribution of all swimming speeds in light. *Tg(hsp:Prok2)* fish spent more time at lower speeds in light compared to their WT siblings ($p=1.7\times10^{-13}$, Kolmogorov–Smirnov test).

Figure S5. *prok2* and *prok receptor* mutants lack development defects associated with Kallmann syndrome, sleep architecture of *prok2* mutant larvae, and *prok2* mutant larvae exhibit normal circadian regulation of locomotor activity and sleep. Related to Figures 5 and 6. (A) Representative genotyping images for *prok2*, *prokr1* and *prokr2* mutants. Band patterns for homozygous mutant, heterozygous mutant and homozygous WT are described in STAR Methods. (B) Images of representative WT, *prok2*-/-, and *prokr1*-/-; *prokr2*-/- 6-month old adult zebrafish. All fish are similar in size and morphology. (C) Representative images of ISH using probes specific for *gnrh2*, *gnrh3*, *th1* and *emx1* in 5 dpf WT, *prok2*-/-, and *prokr1*-/-; *prokr2*-/- larvae. Dashed boxes indicate olfactory region. (D) Representative images of ISH using probes specific for the hypothalamic neuropeptides *hcrt*, *qrfp* and *prok2* in *prok2*+/+, *prok2*+/- and *prok2*-/- larvae. Larvae of all genotypes show similar expression levels and patterns of each gene. There is no apparent nonsense-mediated decay of *prok2* mRNA in *prok2* mutants. Animals were genotyped by PCR after imaging. Scale bars: (B) 5 mm, (C) 100 µm, (D) 10 µm. (E-J) Sleep architecture of *prok2* mutant larvae. The increased activity and decreased sleep during days 6 and 7 of development in *prok2*-/- larvae is due to a decrease in the number of sleep bouts (E), with a trend towards longer waking bouts (I). (K-P) Larvae entrained in 14:10 hour LD conditions for 5 days and then monitored in DD maintain normal circadian rhythms of locomotor activity (K-M) and sleep (N-P), with no significant differences between *prok2*-/-, *prok2*+/- or *prok2*+/+ siblings. Data from 5 experiments (E-J) and 2 experiments (K-P) combined are shown. Bar graphs show mean ± SEM. n = number of larvae. **$p<0.01$ by one-way ANOVA, with post-hoc Dunnett's test compared to WT.

Figure S6. *prok receptor* larval mutants lack sleep/wake phenotypes. Related to Figures 5 and 7. (A, B) Amino acid sequence alignments of human (Hs), mouse (Mm), and zebrafish (Dr) Prokr1 (A) and Prokr2 (B) orthologs, and the sequences of two zebrafish Prokr1 mutant proteins (d7 and d22) and two zebrafish Prokr2 mutant proteins (d1 and d14) that were generated in this study. Predicted transmembrane (TM) domains are indicated by red lines. Both Prokr1 mutant proteins are truncated at the third TM domain. Both mutants exhibit similar phenotypes and mutant d7 was used for all reported experiments. Both Prokr2 mutant proteins are truncated before the first TM domain. Both mutants exhibit similar phenotypes and mutant d1 was used for all reported experiments. (C-H) *prokr1*-/-, *prokr1*+/- and *prokr1*+/+ larvae exhibit similar amounts of locomotor activity (C-E) and sleep (F-H) during the day and night. (I-N). *prokr2*-/-, *prokr2*+/- and *prokr2*+/+ larvae exhibit similar amounts of locomotor activity (I-K) and sleep (L-N) during the day and night. (O-T) *prokr1*+/-;*prokr2*+/-, *prokr1*-/-;*prokr2*+/-, *prokr1*+/-;*prokr2*-/- and *prokr1*-/-;*prokr2*-/- larvae exhibit similar amounts of locomotor activity (O-Q) and sleep (R-T) during the day and night. Data from one experiment (C-I, L) and two experiments (J, K, M-T) combined are shown. Bar graphs show mean ± SEM. n = number of larvae.

Figure S7. The Prok2 overexpression phenotype requires *prokr2* but not *prokr1* or *dbh*. Related to Figure 7. (A-F) Following heat shock at 5 dpf (gray bar in (A, D)), Prok2 overexpression-induced locomotor activity (A-C) and sleep (D-F) phenotypes are abolished in *prokr2*-/- larvae (yellow), but not in *prokr2*+/- larvae (red). Data from two experiments combined is shown. (G-L) Following heat shock at 5 dpf (gray bar in (G, J)), Prok2 overexpression-induced locomotor activity (G-I) and sleep (J-L) phenotypes are similar in *prokr1*-/- (yellow) and *prokr1*+/- larvae (red). Data from two experiments combined is shown. (M-R) *dbh*-/- larvae are less active (M-O) and sleep more (P-R) than their *dbh*+/- siblings during the day and night. Prok2 overexpression at 5 dpf (gray bar in (M, P)) decreased activity and increased sleep during the day (N, Q), and increased activity and decreased sleep at night (O, R), in both *dbh*+/- and *dbh*-/- larvae. Note the lack of difference between *Tg(hsp:Prok2);dbh*+/- and *Tg(hsp:Prok2);dbh*-/- larvae on day 5 post heat shock may be due to a floor effect. The magnitude of difference between *Tg(hsp:Prok2);dbh*+/- and *Tg(hsp:Prok2);dbh*-/- larvae on day 6 is similar to that between *dbh*+/- and *dbh*-/- larvae. Data from one representative experiment (M, P) and two experiments combined (N, O, Q, R) are shown. Bar graphs show mean ± SEM. n = number of larvae. *p< 0.05, **p< 0.01, ***p< 0.001, n.s. p>0.05 by two-way ANOVA, with post-hoc Tukey's HSD test.

Figure S8. Effects of *prok2* gain- and loss-of-function on hypothalamic *galn* expression, and *prok2*-expressing neuron fibers are present in the optic tectum. Related to Figure 8. (A, B) Prok2 overexpression increases hypothalamic *galn* expression in *prokr2*+/- larvae maintained in light for 4 hours after heat shock, but this effect is abolished in *prokr2*-/- larvae. (A) 28.8 μm thick confocal projections from representative brains showing hypothalamic *galn* FISH in *Tg(hsp:Prok2)* and non-transgenic *prokr2*+/- or *prokr2*-/- siblings. Animals were heat shocked at 6 dpf from 2-3 p.m. and then kept in light until fixation 4 hours later. (B) Mean ± SEM total fluorescence intensity of *galn* FISH. At least 7 brains were quantified for each condition. *p<0.05 by two-way ANOVA, with post-hock Tukey's HSD test. (C, D) Prok2 overexpression induces *galn* expression in a light-specific manner. (C) 28.8 μm thick confocal projections from representative brains showing hypothalamic *galn* FISH. *Tg(hsp:Prok2)* and non-transgenic siblings were heat shocked at 6 dpf from 2-3 p.m. and then kept in light until 7 p.m. (4 hr on). Lights were then turned off for 1 hour (5 hr off), on for 1 hour (6 hr on) and off for 1 hour (7 hr off), and samples were fixed at the end of each hour. On and off refer to samples that were exposed to light or dark during the hour before fixation. (D) Mean ± SEM total fluorescence intensity of *galn* FISH. *galn* mRNA levels were only significantly higher in Prok2-overexpressing larvae that were exposed to light during the hour before fixation. At least 9 brains were analyzed for each condition. **p<0.01 by two-tailed Student's *t* test. (E, F) *prok2* mutant larvae have normal hypothalamic *galn* expression levels. (E) 28.8 μm thick confocal projections from representative brains showing hypothalamic *galn* expression in *prok2*+/+, *prok2*+/- and *prok2*-/- larvae that were raised in LD until 6 dpf and fixed at 10 a.m. (day) or 12 a.m. (night). These time points are 1 hour after lights on and off, respectively. (F) Mean ± SEM total fluorescence intensity of *galn* FISH. *galn* mRNA levels are not significantly different among the genotypes during the day or night (p>0.05 by two-way ANOVA). At least 7 brains were analyzed for each condition. (G-J) Fibers from *prok2*-expressing neurons are present in the optic tectum in larval zebrafish. (G) A 20 μm thick confocal projection showing the hypothalamus of a WT larva injected with a *prok2:GFP-Aequorin* transgene, fixed at 5 dpf and analyzed by double FISH using probes specific for *prok2* (red) and *gfp* (green). (G''') shows an orthogonal view of this image at the position of the blue line in (G''), indicating co-localization of signals from the two probes. Arrows indicate examples of cells that express both genes. (H) Quantification showing that 98% of *gfp-aequorin* expressing cells co-express *prok2*, indicating that the transgene is specifically expressed in *prok2*-expressing neurons. Mean ± SEM for 7 animals is shown. (I) A 130 μm thick confocal projection showing the brain of a WT larva injected with the *prok2:GFP-Aequorin* transgene, fixed at 5 dpf and labeled with a GFP-specific antibody. The GFP-labeled neurons project extensively within the hypothalamus. (I') shows an orthogonal view of this image, revealing dorsal projections to the optic tectum. (J) A 10 μm thick confocal section showing GFP-labeled fibers in the optic tectum. Yellow dashed lines outline the left and right optic tectum. Scale bars: (A, C, E) 50 μm, (G) 10 μm, (I) 100 μm. Anterior, posterior and dorsal axes are indicated.