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Neuropeptide Y regulates sleep by modulating noradrenergic signaling

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SUMMARY

Sleep is an essential and evolutionarily conserved behavioral state whose regulation remains poorly understood. To identify genes that regulate vertebrate sleep, we recently performed a genetic screen in zebrafish, and here we report the identification of neuropeptide Y (NPY) as both necessary for normal daytime sleep duration and sufficient to promote sleep. We show that overexpression of NPY increases sleep, whereas mutation of *npy* or ablation of *npy*-expressing neurons decreases sleep. By analyzing sleep architecture, we show that NPY regulates sleep primarily by modulating the length of wake bouts. To determine how NPY regulates sleep, we tested for interactions with several systems known to regulate sleep, and provide anatomical, molecular, genetic and pharmacological evidence that NPY promotes sleep by inhibiting noradrenergic signaling. These data establish NPY as an important vertebrate sleep/wake regulator and link NPY signaling to an established arousal-promoting system.

eTOC Blurb

Based on a genetic screen, Singh et al identify NPY signaling and *npy*-expressing neurons as regulators of zebrafish sleep. They show that NPY promotes sleep by inhibiting noradrenergic signaling, thus linking NPY signaling to an established arousal-promoting system.

Keywords

Sleep; neuropeptide Y; hypothalamus; locus coeruleus; noradrenaline; locomotor activity; arousal; genetics

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AUTHOR CONTRIBUTIONS

DAP and JR performed the genetic screen. CS and DAP conceptualized and designed the experiments, and generated reagents. CS performed the experiments and analyzed the data. CS and DAP wrote the paper with assistance from JR. DAP supervised the project.

INTRODUCTION

Sleep is among most basic needs of living organisms, yet mechanisms that regulate sleep remain poorly understood. Several neuropeptides have been implicated in regulating mammalian sleep [1], including hypocretin [2–4], which promotes wakefulness, and galanin [5–8] and melanin concentrating hormone [9–12], which promote sleep, suggesting that examining additional neuropeptides may identify novel mechanisms that regulate sleep. Identifying these mechanisms using mammalian models has been challenging due to their poor amenability for large-scale screens, although such screens are possible [13]. As an alternative approach, several groups have used behavioral criteria to study sleep-like states in simpler model organisms that are amenable to screens, including *Drosophila* [14–22], *C. elegans* [23–25], and zebrafish [26–30]. In particular, several groups have demonstrated behavioral, anatomical, genetic and pharmacological conservation of sleep between zebrafish and mammals, establishing zebrafish as a vertebrate sleep model [26–28, 30–33]. We previously described a screen for genes whose overexpression affects zebrafish sleep, and reported that the neuropeptide neuromedin U is necessary and sufficient for normal levels of arousal [29]. Here we demonstrate that another neuropeptide identified in the screen, neuropeptide Y (NPY), is necessary for daytime sleep and sufficient to promote sleep.

NPY is widely expressed in the brain and has been implicated in regulating endocrine, behavioral and circadian processes [34], and is perhaps best known for its role in promoting feeding [35–38]. NPY has also been shown to affect sleep, but its role in this behavioral state remains unclear. Several studies showed that injection of *in vitro* synthesized NPY into the rodent brain [39–45] or intravenously in young healthy [46] or depressed [47] humans can induce sleep or reduce locomotor activity. However, other rodent studies reported the opposite effect [48–50]. The basis for these disparate reports is unclear, but may be due to different sites and doses of NPY injection, or the use of *in vitro* synthesized peptide that may vary in different preparations and from endogenous NPY. Understanding the role of NPY in mammalian sleep is also confounded by links between mechanisms that regulate feeding and sleep [48–51]. Indeed, reports of wake-promotion by injected NPY also observed increased feeding [48–50], suggesting that the increased wakefulness may result from increased feeding. *npy* mutant mice exhibit several phenotypes, including increased anxiety, depression-like behavior, and cognitive deficits [52, 53], and are less susceptible to diet-induced obesity [54]. However, an analysis of sleep in these animals and a role for *npy*-expressing neurons in sleep has not been described. As a result, the role of NPY in vertebrate sleep remains unclear.

Here we show that NPY is sufficient to promote sleep in zebrafish, whereas loss of *npy* or *npy*-expressing neurons results in less daytime sleep. We also show that NPY promotes sleep by inhibiting the wake-promoting noradrenergic system, providing a mechanistic basis for sleep regulation by NPY. Together with the requirement of noradrenergic signaling for the wake-promoting function of hypocretin [55, 56], these results suggest that the noradrenergic system integrates neuropeptidergic signals that regulate sleep/wake states.

RESULTS

Overexpression of human NPY reduces locomotor activity and increases sleep in zebrafish

We previously performed a screen to identify genes that affect larval zebrafish sleep [29]. We injected >1200 unique plasmids in which a heat shock-inducible promoter (*hsp*) regulates the expression of genes that encode for secreted proteins into wild-type (WT) zebrafish embryos at the one-cell stage. We used human open reading frames (ORFs) encoding secreted proteins from the hORFeome 3.1 library [57] because there was no resource of zebrafish ORFs. Co-injection of each plasmid with *tol2 transposase* mRNA resulted in incorporation of the *hsp*-regulated transgene into the genome in many cells of each animal and enabled heat shock-induced overexpression [29]. We then compared sleep/wake behaviors in injected animals before and after heat shock and to negative control animals injected with a *hsp:egfp* plasmid. One gene whose overexpression increased sleep at night (Z-score=1.8) encoded human NPY (Figure S1A). Even though zebrafish exhibit high levels of sleep at night, NPY-overexpressing animals were 28% less active and slept 34% more than control animals during the night after heat shock ($P<0.05$ and $P<0.01$, two-tailed Student's *t* test) (Figures S1B–S1G). We observed a similar phenotype during the day before heat shock that did not reach statistical significance, consistent with leaky expression from the *hsp* promoter that often is observed using this transient injection assay, but is not observed using stable transgenic lines [29].

Overexpression of zebrafish NPY reduces locomotor activity and increases sleep in zebrafish

Using reciprocal BLAST searches, we identified a single zebrafish *npy* ortholog, which encodes for a preproprotein that generates a predicted 36 amino-acid mature peptide that is 89% identical to the human and mouse orthologs (Figure S1H). *npy* is widely expressed in the mammalian brain, particularly in the hypothalamus, amygdala, locus coeruleus (LC) and cerebral cortex [58, 59]. Using *in situ* hybridization (ISH) with an *npy*-specific probe, immunostaining for total extracellular signal-regulated kinase (t-ERK), and image registration to the Z-brain atlas [60], we found that *npy* is similarly expressed in several discrete nuclei within the larval zebrafish brain (Figures S1I–S1N and Movie S1). We also observed *npy* expression in the retina (data not shown) but not in other tissues.

To test whether overexpression of zebrafish NPY affects sleep, we generated *Tg(hsp:npy)* zebrafish. *Tg*(*hsp:npy*) animals and their WT siblings had similar amounts of locomotor activity and sleep before heat-shock (Figures 1A–1D). However, following a heat shock at 3 p.m., *Tg(hsp:npy)* animals were 50% less active (Figures 1A and 1B) and slept 111% more (Figures 1C and 1D) than their WT siblings for the rest of the day ($P<0.0001$, two-tailed Student's *t* test). The phenotype resulted from a 230% increase in the number of sleep bouts (Figure 1E) and an 85% decrease in the length of wake bouts (Figure 1G) ($P<0.0001$, two-tailed Student's *t* test), with a smaller decrease in the length of sleep bouts (Figure 1F), and thus is primarily due to fragmentation of the wake state.

The increase in sleep after the heat shock-induced pulse of NPY overexpression dampened by nighttime. A previous study showed that the circadian system inhibits sleep in the evening, when homeostatic sleep drive is high [61], suggesting the circadian system might limit NPY overexpression-induced sleep to the day. To test whether NPY overexpression can also increase sleep at night, we heat shocked animals during the last hour of the day. We found that *Tg(hsp:npy)* animals were 46% less active (Figures S2A–S2C) and slept 54% more (Figures S2D–S2F) than their WT siblings during the night ($P<0.0001$, two-tailed Student's *t* test), similar to the daytime phenotype when NPY overexpression was induced in the afternoon. This phenotype was due to longer sleep bouts (Figure S2H) and shorter wake bouts (Figure S2J), with no change in the number of sleep bouts (Figure S2G). These results suggest that dampening of NPY-induced sleep at night following heat shock in the afternoon is due to declining levels of NPY rather than effects of the circadian clock.

Light affects locomotor activity and sleep in zebrafish [27, 28], as it does in mammals [62]. To determine whether light affects NPY overexpression-induced sleep, we entrained larvae by raising them in 14:10 hour light:dark (LD) conditions for four days, and then transferred them to constant dark before inducing NPY overexpression. NPY-overexpressing animals were 54% less active and slept 80% more than WT siblings during the rest of the subjective day (Figures S2K–S2N) ($P<0.0001$, two-tailed Student's *t* test). This phenotype was due to more sleep bouts and shorter wake bouts, with no change in the length of sleep bouts (Figures S2O–S2Q). Hence, NPY overexpression promotes sleep independent of lighting condition and circadian phase.

Overexpression of NPY increases arousal threshold

Sleep is distinguished from quiet wakefulness by reduced sensory responsiveness [63]. Because NPY overexpression increases sleep, we asked whether it also alters arousal threshold by monitoring responses to mechano-acoustic stimuli. We found that the stimulus intensity at which we observed the half-maximal response (effective tap power 50, ETP_{50}) was 290% higher for *Tg(hsp:npy)* animals than their WT siblings (Figure 1H) ($P<0.05$ by extra sum-of-squares F test). Thus, NPY overexpression increases arousal threshold, consistent with increased sleep. We next asked if NPY overexpression affects arousal in awake and/or sleeping animals by allowing 5 minutes between trials. According to the behavioral definition of sleep, we scored animals as awake if they moved during the minute before a stimulus was delivered. We used stimulus intensities of 2.3, 3.0 and 4.0 arbitrary units, which were lower than the ETP_{50} values of both *Tg(hsp:npy)* and WT animals. NPY-overexpressing animals were less responsive to these stimuli than WT siblings during both awake (Figure 1I) and sleep (Figure 1J) states. These data suggest that NPY overexpression decreases arousal in awake animals and increases sleep depth in sleeping animals.

npy mutant zebrafish are more active and sleep less during the day

We next asked whether endogenous *npy* is required for normal sleep/wake behaviors by using the zinc finger nuclease method to generate zebrafish containing a predicted null mutation in the *npy* open reading frame [64]. We isolated zebrafish containing a 17-nucleotide deletion in the second exon of the *npy* gene [64], which results in a translational frame shift at the beginning of the mature peptide domain (Figure 2A), generating a protein

that lacks the mature peptide domain and thus is likely nonfunctional. Homozygous mutant animals are viable and fertile, and lack obvious developmental defects.

Consistent with the NPY overexpression phenotype, *npy*−/− larvae were 23% more active and slept 36% less during the day than their *npy*+/+ siblings ($P<0.0001$ and $P<0.01$, one-way ANOVA, Holm-Sidak test) (Figures 2B, 2C, 2E and 2F). These effects were due to fewer sleep bouts (Figure 2H) and longer wake bouts (Figure 2L), with no effect on the length of sleep bouts (Figures 2J). Thus, reduced daytime sleep in *npy*−/− animals is due to consolidation of the wake state. We did not observe *npy*−/− phenotypes at night. These data indicate that endogenous *npy* is required for normal daytime sleep amounts.

Microinjection of NPY into the hamster suprachiasmatic nucleus phase shifts the locomotor activity circadian rhythm in constant light (LL) [65, 66], suggesting that NPY may regulate entrainment or expression of circadian rhythms. To test whether endogenous *npy* is required for circadian regulation of locomotor activity and sleep, we tested larvae that were entrained for 4 days in LD, then monitored for 24 hours in LD and then for 48 hours in LL. Absence of *npy* had no obvious effect on the locomotor activity or sleep circadian period length or phase (Figures 3A and 3D). As expected, in LD *npy*−/− animals were more active (Figures 3A and S3A) and slept less (Figures 3D and S3C) than their *npy*+/+ and *npy*+/− siblings during the day, with no phenotype at night. The daytime phenotype was due to fewer sleep bouts and longer wake bouts (Figures S3E and S3I). Following the shift to LL, *npy*−/− animals were more active by 30% and 26% during the subjective day and night, respectively, compared to their *npy*+/+ siblings ($P<0.001$ and $P<0.01$, one-way ANOVA, Holm-Sidak test) (Figures 3A–3C). *npy*−/− larvae also slept ~40% less during the subjective day and night ($P<0.0001$ and $P<0.001$, one-way ANOVA, Holm-Sidak test) (Figures 3D–3F). These phenotypes were primarily due to longer wake bouts (Figures 3K and 3L), although there were also fewer (Figures 3G and 3H) and shorter (Figures 3I and 3J) sleep bouts. These results indicate that *npy* is not required for circadian regulation of locomotor activity or sleep in zebrafish larvae, but rather regulates sleep in a light-dependent manner.

Ablation of *npy*-expressing neurons increases locomotor activity and decreases sleep

As an alternative approach to test the hypothesis that NPY is necessary for normal sleep duration, we ablated *npy*-expressing neurons. To this end, we generated *Tg(npy:kalta4)* zebrafish, in which NPY neurons express an optimized version of the transcriptional activator Gal4 (KalTA4). To verify the specificity of this transgene, we performed double fluorescent ISH (FISH) using probes specific for *npy* and *kalta4*. We observed that *kalta4* is expressed in >80% of *npy*-expressing neurons (>95% for some brain regions), and that >92% of *kalta4*-expressing neurons express *npy* (Figure S5A and Table S1). We mated these fish to *Tg(uas:nfsb-mcherry)* animals [67], resulting in the expression of nitroreductase (nfsb) in *npy*-expressing neurons (Figure 4A). Nitroreductase is a bacterial protein that converts the inert prodrug metronidazole (MTZ) into a cytotoxic DNA crosslinking agent, thus enabling drug-inducible ablation of the targeted cell type [68]. We treated *Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* and *Tg(npy:kalta4)* sibling control animals with MTZ or DMSO vehicle control for 48 hours (from 3 days post-fertilization (dpf) to 5 dpf). MTZ treatment almost completely eliminated mCherry-labeled cells in double transgenic animals

(Figures 4A–4C), indicating loss of most *npy*-expressing neurons. Consistent with these observations, we detected TUNEL labeling in *npy*-expressing neurons in *Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* animals treated with MTZ, but not in those treated with DMSO (Figures S5B–S5D), indicating that MTZ treatment induces apoptosis of *npy*-expressing neurons. Consistent with the *npy*−/− phenotype, *npy*-ablated animals were 23% more active (Figures 4C and 4D) and slept 28% less (Figures 4F and 4G) ($P<0.01$ and $P<0.05$, two-tailed Student's *t* test) compared to sibling controls during the day. This phenotype was due to fewer sleep bouts (Figure 4I) and longer wake bouts (Figure 4M), indicating consolidation of the wake state, similar to *npy*−/− animals. To confirm that the *Tg(uas:nfsb-mcherry)* transgene alone does not cause a behavioral phenotype, we crossed *Tg(npy:kalta4)/+;Tg(uas:nfsb-mcherry)/+* to WT fish, excluded animals that were positive for mCherry, and treated the remaining animals with MTZ. We observed no difference in locomotor activity or sleep among animals of these three genotypes (Figure S4). The cell ablation phenotype was slightly weaker than that of the *npy* mutant, likely because the *npy:kalta4* transgene is not expressed in all *npy*-expressing neurons. Because a small number of neurons express *kalta4* but not *npy* in some brain regions (8% in the subpallium, <5% in other brain regions; Figure S5A and Table S1), it is possible that ablation of these NPY-negative cells is responsible for the behavioral phenotype. However, this is unlikely to be the case due to the small number of cells involved and because the NPY neuron ablation phenotype is consistent with the *npy* mutant phenotype, suggesting that both NPY and *npy*-expressing neurons are necessary for normal daytime sleep amount.

The NPY overexpression phenotype is not blocked by manipulation of several pathways known to regulate sleep

To identify genetic mechanisms through which NPY affects sleep, we tested whether the NPY overexpression phenotype is suppressed in zebrafish containing mutations in other genes implicated in regulating sleep (Table S2). We found that the NPY overexpression phenotype persisted in larvae containing null mutations in *histidine decarboxylase* (*hdc*) [69], *hypocretin receptor* (*hcrtr*) [27], *corticotropin releasing hormone a* (*crha)* (Singh et al., unpublished), *crhb* (Singh et al., unpublished) or arylalkylamine *N-acetyltransferase 2* (*aanat2*) [70] (data not shown). These data suggest that NPY promotes sleep via other mechanisms.

NPY promotes sleep by inhibiting noradrenergic signaling

Pharmacological and genetic studies in mammals and zebrafish have shown that norepinephrine (NE) plays an important role in promoting arousal [56, 71], and the LC is the primary source of NE in the brain [72]. We obtained several lines of evidence suggesting that NPY promotes sleep by inhibiting NE signaling. First, a nucleus of 3–5 *npy*-expressing neurons is located adjacent to, and sends projections that form close contacts with, LC neurons (Figures 5A–5H and Movie S2). While this does not prove a direct interaction between the two neuronal populations, it is consistent with our functional evidence that NPY promotes sleep by inhibiting NE signaling (see below). The zebrafish genome contains seven annotated *npy receptor* genes [73]. Using FISH, we did not detect *npy receptor* expression in LC neurons, although we observed expression of *npy receptor y1* (*npy1r*) (Figure 5I) and *npy receptor y2 like* (*npy2rl*) (Figure 5J) near the LC. The other *npy*

receptors showed expression in other brain regions (*npy8ar* and *npy8br*) or no detectable pattern of expression (*npy2r*, *npy4r* and *npy7r*) (data not shown). These results suggest that NPY indirectly affects NE signaling, although a *npy receptor* might be expressed in LC neurons at levels too low to be detected using FISH, a common problem for G-protein coupled receptors (GPCRs).

Second, we found that the sedating effects of NPY overexpression and loss of NE signaling are not additive. We made this observation by overexpressing NPY in larvae that lack NE synthesis due to mutation of *dopamine beta hydroxylase* (*dbh*) [56], or that lack NE signaling due to treatment with the α-1-adrenergic receptor antagonist prazosin. Both genetic and pharmacological inhibition of NE signaling increase sleep in zebrafish [56]. If NPY promotes sleep by inhibiting NE signaling, then overexpression of NPY should not further increase sleep in *dbh*−/− larvae or in WT larvae treated with prazosin. Alternatively, if NPY promotes sleep via a NE-independent mechanism, then the combined effects of NPY overexpression and loss of NE signaling on sleep should be additive. Because the behavior of *dbh*+/− animals is indistinguishable from that of their *dbh*+/+ siblings [56], we compared *dbh*+/− and *dbh*−/− siblings to reduce the number of comparisons in each experiment, and thus increase the number of animals per condition. Prior to heat shock-induced NPY overexpression, *dbh*−/− larvae were 40% less active and slept >100% more than their *dbh*+/− siblings for both *Tg(hsp:npy)* animals and their non-transgenic siblings (Figures 6A–6D) ($P<0.01$, two-way ANOVA, Holm-Sidak test). NPY overexpression decreased locomotor activity by 54% and increased sleep by 60% in *Tg(hsp:npy);dbh+/−* animals compared to *dbh+/−* siblings (Figures 6A–6D) ($P<0.0001$ and $P<0.05$, Two-way ANOVA, Holm-Sidak test). However, overexpression of NPY did not further affect the sleep/wake behavior of *dbh−/−* animals, as activity and sleep amounts were indistinguishable for *Tg(hsp:npy);dbh*−/− and *dbh*−/− animals (Figures 6A–6D). We obtained similar results for NPY overexpression in prazosin-treated animals compared to DMSO vehicle-treated controls (Figures S6A–S6D). To confirm that the failure of NPY overexpression to enhance sleep in *dbh*−/− or prazosin-treated animals is not due to a ceiling effect for sleep, we found that treatment with melatonin, an alternative sedative, enhanced sleep induced by overexpression of NPY (Figures S7A–S7D) or prazosin (Figures S7E–S7H).

Third, we found that the increased locomotor activity and reduced sleep observed in *npy*−/− animals compared to their *npy*+/+ siblings was abolished by treatment with prazosin. We made this observation by treating *npy*+/+, *npy*+/− and *npy*−/− larvae with either DMSO or prazosin. If NPY promotes sleep by inhibiting NE signaling, then loss of NPY should not affect prasozin-induced sleep. Alternatively, if NPY promotes sleep via a NE-independent mechanism, then loss of NPY should affect sleep amount in prazosin-treated animals. Consistent with the former possibility, we found that prazosin decreased activity and increased sleep, and this phenotype was indistinguishable for *npy*+/+, *npy*+/− and *npy*−/− siblings (Figures 6E–6J).

Fourth, we found that NPY regulates *dbh* expression in the LC. NPY overexpression decreased *dbh* mRNA in the LC by 38% at 3 hours post-heat shock in *Tg(hsp:npy)* animals compared to WT siblings ($P<0.05$, two-tailed Student's *t* test) (Figures 7A and 7D). This time point coincides with the maximal effect of NPY overexpression on locomotor activity

and sleep (Figures 1A and 1C), suggesting that NPY overexpression-induced sleep may result from reduced *dbh* expression, and thus reduced NE levels. However, effects of NPY overexpression on behavior begin within the first hour after heat shock, and we only observed a trend of decreased *dbh* mRNA at 1 and 2 hours post-heat shock that was not statistically significant (Figure 7D). These observations suggest that reduced *dbh* expression may not be the primary cause of NPY overexpression-induced sleep, but may rather be a secondary effect that maintains NPY-induced sleep, perhaps resulting from decreased LC neuron activity. We also tested whether NPY overexpression affects the level of *tyrosine hydroxylase* (*th*), which acts upstream of *dbh* in the NE synthesis pathway. We found that NPY overexpression did not significantly change *th* mRNA expression in the LC at 1, 2 or 3 hours post-heat shock (data not shown). Reduced *dbh* expression was not simply a consequence of increased sleep, as *dbh* mRNA level was unaffected following overexpression of the sleep-promoting neuropeptide prokineticin 2 (Prok2) [74] (Figure 7E) or treatment with the sedative melatonin (Figure 7E). The interaction between NPY and *dbh* appears to be specific, as NPY overexpression did not affect expression of other genes involved in promoting arousal, including the neuropeptides *hypocretin* (*hcrt*) [28, 56] or *adenylate cyclase activating polypeptide 1a* (*adcyap1a*) (Singh and Prober, unpublished) (Figures 7B, 7C and 7E). These results indicate that overexpression of NPY selectively decreases the level of *dbh* mRNA in the LC, presumably resulting in decreased NE levels and thus increased sleep. In support of this finding, we observed that *dbh* mRNA level was 33% higher in the LC of *npy*−/− animals compared to their *npy*+/− and *npy*+/+ siblings during the day (Figures 7F and 7G) ($P<0.05$, one-way ANOVA, Holm-Sidak test). Moreover, *dbh* mRNA level in the LC of WT animals was 25% lower at night compared to the day ($P<0.05$, two-tailed Student's *t* test) (Figures 7H). This result demonstrates a correlation between the wake circadian phase of this diurnal species and the level of *dbh* mRNA in the LC, and suggests that changes in NE levels contribute to the regulation of normal sleep/wake states. Taken together, these results are consistent with a model in which NPY promotes sleep by inhibiting NE signaling.

DISCUSSION

NPY has been shown to affect sleep in mammals, but its role in sleep has been unclear. Infusion of NPY in rodents has been reported to increase [39–45] or decrease [48–50] sleep. These opposite effects may be due to different sites of injection or dosage, or the use of *in vitro* synthesized NPY that may lack modifications present on endogenously produced peptide. These studies are also confounded by other functions of NPY. For example, experiments in rats found the wake-promoting effects of NPY to be associated with feeding behaviors [48–50], and NPY can induce hypothermia [75] and increase social interactions [76], which may affect sleep. In agreement with some rodent studies, intravenous NPY injection promoted sleep in both healthy [46] and depressed [47] humans. Reduced NPY was observed in humans with major depression who report sleep disturbances [77] and in humans with primary insomnia [78], consistent with a sleep-promoting role for NPY. Reduced NPY was also found in individuals with post-traumatic stress disorder (PTSD) [79] and could contribute to the insomnia and fragmented sleep experienced by these patients. *npy*-expressing neurons are also implicated in mammalian sleep. For example, GABAergic

cortical interneurons co-expressing *neuronal nitric oxide synthase* (*nnos*) and *npy* express *c-fos*, a marker of neuronal activity, during sleep in rodents [80]. Furthermore, extracellular single-unit activity in the basal forebrain of anaesthetized rats showed increased firing of *npy*-expressing neurons during slow wave sleep [81].

To address the role of endogenous NPY in sleep, we performed genetic gain- and loss-of-function studies using zebrafish larvae. These studies are performed before the onset of feeding, when larvae receive nutrients from the yolk sac [82], and before the onset of social interactions [83]. Furthermore, because zebrafish are poikilothermic, thermoregulation is unlikely to be a factor in studies of zebrafish sleep. Thus, zebrafish larvae allow the role of NPY in sleep to be addressed without complications of mammalian models. We found that overexpression of NPY suppresses locomotor activity and increases sleep during the day and night, whereas *npy* mutant zebrafish are more active and sleep less during the day. Analysis of sleep architecture revealed that NPY overexpression results in shorter wake bouts, whereas *npy* mutants have longer wake bouts, suggesting that NPY regulates consolidation of the wake state. Consistent with this phenotype, ablation of *npy*-expressing neurons resulted in decreased sleep during the day, again due to longer wake bouts. The daytime specificity of the loss-of-function phenotype could be explained by the presence of redundant sleep-promoting systems at night, the primary sleep phase of zebrafish. Consistent with our observations, overexpression in *Drosophila* of neuropeptide F (NPF), a *Drosophila* homolog of NPY, or its receptor NPFR1, promotes sleep [84], although stimulation of NPF neurons was recently shown to promote wakefulness and feeding [85]. This discrepancy could arise from differences in nutritional status [85]. The *Drosophila* short neuropeptide F (sNPF) is also thought to promote sleep [86] and has been referred to as an NPY ortholog, but is more likely an ortholog of vertebrate RFamide peptides [87]. In *C. elegans*, locomotor quiescence during lethargus is abolished in mutants lacking the receptor *npr-1* and reduced in mutants lacking the *npr-1* ligands *flp-18* and *flp-21* [88]. *npr-1* mutants are also more responsive to oxygen and pheromones, resulting in altered foraging and accelerated locomotion [89–91]. While NPR-1 is related to NPY receptors [92], FLP-18 and FLP-21 are more similar to RFamide peptides [87, 93]. Combined with our results, these studies establish NPY as a conserved sleep promoting neuropeptide, and the human studies described above suggest this function is conserved in humans.

npy is widely expressed in the mammalian brain, particularly in the hypothalamus, amygdala, LC and cerebral cortex [58, 59]. Similar to mammals, NPY is expressed in several discrete brain regions in zebrafish larvae. Because of this broad expression pattern, NPY could act via several known sleep/wake regulators. First, *npy*-expressing neurons innervate *hcrt*-expressing neurons, and NPY inhibits *hcrt* neurons in mouse brain slices [94]. Second, a hypothalamic population of *npy*-expressing neurons project to the histaminergic tuberomammillary nucleus in rodents [95]. Third, corticotropin releasing hormone (CRH) impairs sleep and enhances vigilance [96], and NPY enhances inhibitory synaptic transmission in *crh*-expressing neurons in amygdala brain slices [97]. Fourth, melatonin promotes sleep in diurnal vertebrates, including humans [98], and application of NPY to rat pineal explants increases melatonin production [99]. To determine whether any of these pathways underlie the sleep-promoting effects of NPY, we tested whether the NPY overexpression phenotype is blocked in zebrafish mutants in which these pathways are

affected, but found this not to be the case. We also found that NPY overexpression increased sleep in WT and melatonin-treated animals to a similar extent. These observations suggest that NPY does not affect sleep by modulating these pathways.

In contrast to these negative results, we made several observations suggesting that NPY promotes sleep by inhibiting NE signaling. Pharmacological and genetic studies in mammals and zebrafish have shown that NE promotes arousal, and that inhibition of NE signaling increases sleep [56, 71, 72]. We found that overexpression of NPY did not enhance the increased sleep observed in *dbh*−/− animals and prazosin-treated WT animals, suggesting that NPY overexpression promotes sleep by inhibiting NE signaling. Consistent with this possibility, we found that prazosin treatment abolished the decreased sleep observed in *npy* mutants, suggesting that elevated NE signaling underlies the *npy* mutant phenotype. In support of these functional interactions, we found that NPY overexpression decreases the level of *dbh* mRNA in the LC, the primary source of NE in the brain [72], and thus likely reduces NE levels. We observed a trend of reduced *dbh* mRNA levels at 1 and 2 hours after induction of NPY overexpression, and a significant reduction at 3 hours post-heat shock. These observations suggest that reduced *dbh* expression may not be the primary cause of NPY overexpression-induced sleep, but rather may be a secondary effect that maintains NPY-induced sleep, perhaps resulting from decreased LC neuron activity. Consistent with this possibility, NPY can inhibit LC neurons in rodent brain slices [100]. However, the maximal effect of NPY overexpression on behavior occurred at ~3 hours post-heat shock, coinciding with a significant reduction in *dbh* expression in the LC, consistent with NPY directly promoting sleep by decreasing *dbh* expression, and thus NE production, in the LC. Moreover, we found that *npy* mutants have elevated *dbh* expression in the LC, presumably resulting in increased NE levels and increased arousal. It was recently shown that *dbh* expression undergoes a circadian oscillation in whole zebrafish larvae [101]. Consistent with this observation, we found that the level of *dbh* mRNA in the LC is lower at night compared to the day, suggesting that NE levels contribute to the diurnal sleep/wake cycle.

Consistent with an interaction between NPY and the LC, we identified a small population of *npy*-expressing neurons that is adjacent to, and appears to innervate, the LC. This observation contrasts with mammals, where *npy* and *dbh* are co-expressed in LC neurons [102, 103]. We were unable to detect expression of NPY receptors in LC neurons, suggesting that NPY may indirectly affect NE signaling. However, expression of GPCRs, the protein class of NPY receptors, is notoriously difficult to detect, and we thus cannot rule out the possibility that a NPY receptor is expressed in LC neurons. We did observe expression of *npy1r* and *npy2rl* in cells near the LC, suggesting the possibility of local indirect interactions between NPY neurons and the LC. Thus, while the anatomic interaction between the NPY and NE systems appears to differ in zebrafish and mammals, the functional relationship between the systems may be conserved. Taken together, these observations suggest that NPY could regulate sleep by directly affecting the firing of LC neurons and/or the level of NE. Alternately, the site of interaction between NPY and NE in sleep may lie in a network of neurons near the LC or elsewhere in the brain.

In both mammals and zebrafish, NE is necessary for the wake-promoting functions of Hcrt signaling and *hcrt*-expressing neurons [55, 56]. Here we provide evidence that NE signaling

mediates the sedating effect of NPY, suggesting a central role for the NE system in neuropeptidergic regulation of sleep/wake states. While Hcrt and NPY have opposite effects on sleep via NE signaling, both neuropeptides promote feeding via neurons in the hypothalamus [38, 104], suggesting a segregation of neuronal circuits through which these neuropeptides regulate sleep and feeding. While an interaction between NPY and the LC has been shown to control stress responses in rodents [105], to our knowledge this is the first demonstration of an interaction between NPY and the NE system in the context of sleep.

Finally, we note that cerebrospinal fluid levels of NPY are reduced in individuals suffering from PTSD who have sleep disturbances [79], and treatment with prazosin reduces nightmares and improves sleep in these individuals [106]. Since we found that *npy* mutant zebrafish have elevated *dbh* expression, and presumably more NE, the reduced NPY observed in in PTSD might cause increased NE levels, thereby disrupting sleep. These observations suggest that NPY might be therapeutic for at least some aspects of PTSD.

In summary, our results identify NPY as a regulator of sleep/wake behaviors in zebrafish and suggest that NPY promotes sleep by inhibiting NE signaling. These results highlight a central role for NE signaling in regulating sleep, and suggest that modulation of NPY signaling may be a useful therapeutic approach for sleep disorders.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by, the Lead Contact David A. Prober (dprober@caltech.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish experiments and husbandry followed standard protocols [107] in accordance with Caltech Institutional Animal Care and Use Committee guidelines. Larval zebrafish were studied before the onset of sexual differentiation and all behavioral experiments were performed using siblings with the same genetic background, differing only in the presence of a transgene, mutation of a specific gene, or treatment with drugs and appropriate vehicle controls. The age of animals used in each experiment is described in the manuscript, in each figure legend, and/or in the STAR Methods.

Transgenic and mutant zebrafish

***Tg(hsp:npy)* ct853Tg:** Full-length zebrafish *npy* cDNA was isolated using 5′ and 3′ RACE (FirstChoice RLM-RACE, AM1700, Thermo Fisher Scientific) and the open reading frame was cloned downstream of the zebrafish *hsp70c* promoter [28] in a vector containing flanking I-SceI endonuclease recognition sites. The same zebrafish *npy* gene was cloned in a previous study [108], but the gene isolated in our study contains an arginine residue located C-terminal to the mature peptide domain that was reported as an alanine residue in the previous study [108]. The sequence reported in our study is the same as that reported by the zebrafish genome sequencing project (www.ensembl.org/Danio_rerio). The alanine residue described in the previous report [108] is therefore likely either a sequencing error or a

polymorphism in the fish strain used. Stable transgenic lines were generated by injecting plasmids with I-SceI (R0694, New England Biolabs Inc.) into zebrafish embryos at the one-cell stage. Transgenic founders were identified by outcrossing potential founders, heat shocking progeny at 5 dpf, fixing animals 30 minutes after heat shock and performing ISH using an *npy*-specific probe. *Tg(hsp:npy)* fish were genotyped using the primers 5′-CCGCCACCATGAATCCA-3′ and 5′-GGTTTGTCCAAACTCATCAATGT-3′, which generate a 370 bp band. We generated two independent *Tg(hsp:npy)* stable transgenic lines that produced similar phenotypes, but all data shown in the paper are from the line that produced stronger phenotypes.

***npy* mutant ct811:** *npy* mutant zebrafish were generated using the zinc finger nuclease method [64]. The mutant contains a 17 bp deletion (AGCCCGACAACCCGGGA) after nucleotide 94 of the open reading frame, resulting in a translational frame shift beginning at the fourth amino acid of the mature peptide domain. Mutant animals were genotyped using the primers 5′-ATAAATTGCGCATCAGCACA-3′ and 5′-TGAGGAAGAATTTGAGACTACGC-3′, which produce a 281 or 264 bp band for the WT or mutant allele, respectively. *npy* heterozygous mutants were outcrossed to the parental TLAB strain for four generations before use in behavioral experiments. Homozygous *npy* mutants are viable, fertile, lack obvious developmental defects and are morphologically indistinguishable from WT animals.

***Tg(npy:kalta4)* ct852Tg:** We used bacterial artificial chromosome (BAC) recombineering [109] to insert an optimized version of the transcriptional activator Gal4 (KalTA4) [109] at the *npy* start codon of a BAC (zK50N10SP6; HUKGB735N1050Q, Source BioScience)) containing 288 kb of genomic sequence, including 145 kb upstream and 143 kb downstream of the *npy* gene. Primers of 70 nucleotides (pIndigoBAC_HA1_iTol2_F and pIndigoBAC_HA1_iTol2_R, Table S3) were used to amplify the long terminal repeats of the medaka Tol2 transposon to enable single-copy integration of the BAC into the zebrafish genome, using the plasmid *pIndigoBAC-536* [109] as template. *npy*-specific primers were designed that contain 50 nucleotide homology arms around the *npy* start codon (positions −53 to −4 and +4 to +53) with ~20 nucleotide ends (Homology arm F and Homology arm R, Table S3 to amplify a KalTA4_kanamycin cassette from the plasmid *pCS2+_kalta4_kanR* [109]. These plasmids were a kind gift from Dr. Stefan Schulte-Merker. The modified BAC was purified using the Nucleobond BAC 100 kit (740579, Macherey-Nagel) and injected into zebrafish embryos at the one- or two-cell stage at a concentration of 50 ng/μL, along with *tol2 transposase* mRNA at a concentration of 50 ng/μL. Transgenic lines were identified by mating potential founders to WT TLAB fish, and progeny were genotyped using the primers 5′-CGCTATCATTTATAGATTTTTGCAC-3′ and 5′-AGTAGCGACACTCCCAGTTG-3′, which produce a 220 bp band in transgenic animals. Transgenic founders were crossed to the *Tg(uas:nfsb-mcherry)* line [67] and the strongest line was identified by fluorescence microscopy.

Other transgenic and mutant lines: The *Tg(dbh:EGFP)* transgenic line [110], *dbh* mutant [56], *hcrtr* mutant [27], *hdc* mutant [69], and *aanat2* mutant [70] have been previously described. The *crha* and *crhb* mutants are unpublished (Singh and Prober unpublished).

METHOD DETAILS

Locomotor activity assay—At 4 dpf, individual larvae were placed into each well of a 96-well plate (7701–1651, GE Healthcare Life Sciences) 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). Plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation, except in experiments where drugs were added. The sealing process introduces air bubbles in some wells, which are excluded from analysis. In experiments using transgenic animals, larvae were blindly assigned a position in the plate, and were genotyped after the behavioral experiment was completed. 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 fixed-angle megapixel lens (M5018-MP, Computar) and infrared filter. For heat shock-induced overexpression experiments, larvae were heat shocked at 37°C for 1 hour starting at either 3 p.m. or 10 p.m. at 5 dpf. The movement of each larva was captured at 15 Hz and recorded using the quantization mode in 1-minute time bins. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared LEDs, and illuminated with white LEDs from 9 a.m. to 11 p.m., except as noted in constant light or constant dark experiments. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5°C. The parameters used for detection were: detection threshold, 15; burst, 29; freeze, 3, which were determined empirically. Data were processed using custom PERL and Matlab (The Mathworks, Inc.) scripts, and statistical tests were performed using Prism 6 (GraphPad).

A movement was defined as a pixel displacement between adjacent video frames preceded and followed by a period of inactivity of at least 67 ms (the limit of temporal resolution). Any one-minute period with no movement was defined as one minute of sleep based on arousal threshold changes [28]. A sleep bout was defined as a continuous string of sleep minutes. Average activity was defined as the average amount of activity in seconds/hour, including sleep bouts.

Arousal threshold assay—The arousal threshold assay was performed as described [56]. Animals were heat shocked at 5 dpf from 12 p.m. to 1 p.m, and taps of 14 different intensities were applied in a random order from 3 p.m. to 10 p.m. Thirty trials were performed at each stimulus intensity, with a 1-minute inter-trial interval. The background probability of movement was calculated by identifying for each genotype the fraction of larvae that moved 5 seconds prior to all stimuli delivered. This value was subtracted from the average response fraction value for each tap event. A response is defined as any movement that occurred within 1 second after a tap was delivered. Data was analyzed using Matlab (Mathworks, Inc.) and dose-response curves were constructed using the Variable Slope log(dose) response curve fitting module of Prism (Graphpad) and fitted using ordinary least squares. The effective tap power 50 (ETP_{50}) was defined as the tapping intensity at which 50% of the maximum number of responding larvae occurs, based on the fitted curve.

Tapping experiments with a 5-minute inter-trial interval were performed using three tap intensities of 2.3, 3.0 and 4.0 arbitrary units to assess the response of awake and sleeping

larvae to the stimuli. These stimulus intensities were chosen because they were lower than the ETP_{50} of animals of both genotypes. Animals were heat shocked at 5 dpf from 12 p.m. to 1 p.m., and thirty-three trials were performed at each stimulus intensity in a random order from 3:00 p.m. to 10:30 p.m. Behavioral responses were analyzed as described above. Three independent experiments for were performed for both 1-minute and 5-minute tapping assays, and one representative experiment for each is shown.

***In situ* hybridization (ISH)—**Animals were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 16 hours at room temperature. ISH was performed using digoxygenin (DIG) labeled antisense riboprobes (DIG RNA Labeling Kit, 11175025910, Sigma-Aldrich), followed by incubation with a sheep anti-digoxigenin-POD antibody (1:400; 11207733910, Sigma-Aldrich), and developed using the TSA Plus Fluorescein and Cyanine 3 System (NEL753001KT, PerkinElmer). Double-fluorescent ISH was performed using DIG- and fluorescein-labeled riboprobes (Fluorescein RNA Labeling kit, 11685619910, Sigma-Aldrich), and the TSA Plus Fluorescein and Cyanine 3 System (NEL753001KT, PerkinElmer) using a previously described protocol [28]. Probes specific for *npy*, *dbh*, *adcyap1a*, *kalta4*, *npy1r*, *npy2r*, *npy2rl*, *npy4r*, *npy7r*, *npy8ar* and *npy8br* were synthesized using standard protocols [111]. The *npy* probe was transcribed using a PCR product amplified from a zebrafish cDNA library using the primers Forward: 5′-CCACAGAGCAAGAATTCCAA-3′ and Reverse: 5′-CAGTCATTATTGTTCTCCTTTGC-3′, and then serially amplified with the same Forward primer and the Reverse Primer with a T7 promoter sequence added: 5′-TAATACGACTCACTATAGGGCAGTCATTATTGTTCTCCTTTGC-3′. The *kalta4* probe was transcribed using the plasmid *pCS2+_kalta4_kanR* [109] as a template after linearization with BamH1 and using T7 RNA polymerase (10881767001, Sigma-Aldrich). A probe specific for *dbh* has been previously described [112]. Probes specific for *adcyap1a*, *npy1r*, *npy2r*, *npy2rl*, *npy4r*, *npy7r*, *npy8ar* and *npy8br* were generated as described for the *npy*-specific probe using the primers listed in Table S3.

Immunohistochemistry (IHC)—Samples were fixed in 4% PFA in PBS overnight at 4°C and then washed with 0.25% Triton X-100/PBS (PBTx). Brains were manually dissected and blocked for at least 1 hour in 2% goat serum/2% dimethyl sulfoxide (DMSO)/PBTx at room temperature or overnight at 4°C. Primary antibody incubations were performed in blocking solution overnight at 4°C using chicken anti-GFP (1:400, GFP-1020, Aves L abs, Inc.) and rabbit anti-DsRed (1:100, 632496, Clontech Laboratories, Inc.). Secondary antibody incubations were performed in blocking solution overnight at 4°C using Alexa Fluo r 488 goat anti-chicken (1:500, A-11039, Thermo Fisher Scientific) and Alexa Fluor 568 goat anti-rabbit (1:500, A-11011, Thermo Fisher Scientific) antibodies. Samples were mounted in 50% glycerol/PBS and imaged using a Zeiss LSM 780 confocal microscope with a 25× 0.8 NA water immersion objective (LD LCI Plan-Apochromat 25x/0.8 1mm Corr DIC M27). Images were processed using Fiji [113].

Z-brain registration—WT larvae were fixed at 6 dpf and ISH was performed using an *npy*-specific probe on dissected brains as described above, followed by IHC using mouse anti-t-ERK primary antibody (1:500, 4696, Cell Signaling Technology) and Alexa Fluor 488

goat anti-mouse secondary antibody (1:500, A32723, Thermo Fisher Scientific). Imaging was performed using a Zeiss 780 confocal microscope, using a 20× 1.0 NA water dipping objective (W Plan-Apochromat 20x/1.0 DIC CG=0.17 M27 75mm) and imaged at ~0.8/0.8/2 μm voxel size (x/y/z) using the Zeiss tiling function and the pairwise stitching function of Fiji [113]. Non-rigid image registration was performed using the Computational Morphometry Toolkit (CMTK, http://www.nitrc.org/projects/cmtk/) as previously described [60]. t-ERK staining was used to register to the t-ERK reference brain [60], which was then used to align *npy* ISH labeling. Registered brains were analyzed using the Z-Brain browser (MATLAB) [60] to identify anatomical regions expressing *npy*. Using Fiji, the registered brain showing *npy* expression was merged to the database 'Anti-tERK_6dpf_MeanImageOf193Fish' from 'AnatomyLabel DatabaseDownsampled' from the Z-Brain Downloads [60] to show the expression of *npy* relative to t-ERK in the reference 6 dpf zebrafish larva. The combined stack was converted into a movie and processed in Windows Movie Maker to add anatomical labels.

Image processing in Imaris and Fiji—Surface rendering to reconstruct projections of *npy*- and *dbh*-expressing neurons was performed using Imaris 9 (Bitplane). To perform surface rendering, we used the Volume function followed by the Normal Shading mode to add a depth effect to the 2-dimensional z-stack imaged using a 63× 1.4 NA oil immersion objective (Plan-Apochromat 63x/1.4 oil DIC M27), and then displayed the image in the 3-dimensional isometric view. We then used the Interactive Software Histogram to select a threshold that included as much of the neuronal projections as possible while excluding any background. Areas of overlap between projections from *npy*- and *dbh*-expressing neurons were magnified 4-fold and saved as TIFF images.

To identify the sources of overlapping projections, a 63x z-stack of *npy*-expressing and *dbh*-expressing neurons was converted to an 8-bit stack. Projections from a single *npy*-expressing neuron and a single *dbh*-expressing neuron were manually traced using the Simple Neurite Tracer plugin in Fiji. Tracings were then filled-in using the same plugin, with an exemplar *npy*-expressing neuron labeled magenta and an exemplar *dbh*-expressing neuron labeled green, and saved as individual z-stacks. These z-stacks were then merged with the original z-stack to so that the traced *npy*-expressing and *dbh*-expressing neurons were overlaid on the original images. As a result, the traced *npy*-expressing neuron appears magenta and the traced *dbh*-expressing neuron appears yellow. This merged image stack is shown in Movie S2.

TUNEL staining—*Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* larvae were treated with DMSO or 10 mM MTZ for 18 hours starting at 3 dpf, and then were fixed in 4% PFA in PBS for 16 hours at 4°C, and subjected to a TUNEL Assay (*In Situ* Cell Death Detection Kit, 11684795910, Sigma-Aldrich) according to the manufacturer's instructions.

Analysis and quantification of *dbh* expression using ISH—*dbh* ISH was performed by incubating fixed 5 dpf brains with a DIG-labeled *dbh* antisense riboprobe, followed by a sheep anti-digoxigenin-POD antibody (1:400; 11207733910, Sigma-Aldrich), and developed using the TSA Plus Cyanine 3 System (NEL753001KT, PerkinElmer). Samples were developed using the cyanine 3 substrate at 1:300 for 5 minutes to avoid

saturation. Brains were imaged using a Zeiss LSM 780 confocal microscope using a 561 nm laser and a 25× 0.8 NA water immersion objective (LD LCI Plan-Apochromat 25×/0.8 1mm Corr DIC M27). To quantify *dbh* expression in *Tg(hsp:npy)* animals, larvae were heat shocked from 3 p.m. to 4 p.m. and samples were collected at the indicated times after heat shock. To quantify *dbh* expression in *npy* mutants, samples were collected at 4 p.m. Both experiments used siblings whose brains were processed for ISH in the same tube, imaged, quantified and then genotyped by PCR. To compare *dbh* expression levels during the day and night, day samples were collected at 4 p.m. and night samples were collected at 2 a.m. After fixation, a small nick was made in the forebrain of night samples to enable their identification at the end of the experiment. Day and night samples were then placed together in the same tube, processed for ISH, imaged and then quantified. Three independent experiments were performed and images of representative samples are shown. For quantification of *dbh* mRNA level, confocal z-stacks were obtained as described above. Using Fiji [113], each z-stack was converted into a maximum intensity projection, converted into 8-bit grayscale, and thresholded to select only the fluorescent ISH signal. This function was applied to all images in an experiment to determine a threshold level that was optimal for most images, and this threshold was then used for all images in an experiment. The Analyze-Set Measurements function was used to select Integrated Density as the measurement parameter and Limit to Threshold was selected to measure only the thresholded region. Fluorescent intensity was then measured by the Analyze-Measure function.

QUANTIFICATION AND STATISTICAL ANALYSIS

All line graphs show a 1 hour forward moving average plotted in 10 minute bins, except Figures S1B and S1E, which show data plotted in 10 minute bins. Line and bar graphs show mean ± standard error of the mean (SEM). In all statistical tests, the significance threshold was set to $P<0.05$. Parametric statistical tests were used because the data followed an approximately normal distribution. For behavioral experiments that compared two genotypes, statistical significance was assessed using a two-tailed Student's *t* test. For *npy* mutant experiments, which compared animals of three different genotypes, one-way ANOVA followed by the Holm-Sidak correction for multiple comparisons was performed to test for significant pair-wise comparisons among all genotypes. The Holm-Sidak test was used to focus on significance but not confidence intervals. For experiments in which NPY was overexpressed in various mutant backgrounds or in which NPY overexpression was combined with drug treatments, statistical significance was assessed using two-way ANOVA followed by the Holm-Sidak correction for multiple comparisons. For experiments in which *npy* mutants were treated with drugs, statistical significance was assessed using two-way ANOVA followed by Holm-Sidak correction for multiple comparisons. For quantification of ISH data, statistical significance was assessed using a two-tailed Student's *t* test for experiments that compared two samples, and one-way ANOVA followed by the Holm-Sidak correction for multiple comparisons for experiments that compared three or more samples. Behavioral data was processed using Matlab (MathWorks), graphs were generated using Excel (Microsoft), and statistical analyses were performed using Prism 6 (Graphpad). The number of animals and statistical test used are stated in each figure or figure legend.

DATA AND SOFTWARE AVAILABILITY

Custom PERL and MATLAB code used for zebrafish behavioral analysis is available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- A genetic screen in zebrafish shows that overexpression of NPY promotes sleep
- Mutation of *npy* or ablation of *npy*-expressing neurons results in decreased sleep
- NPY regulates sleep primarily by modulating the length of wake bouts
- NPY promotes sleep by inhibiting noradrenergic signaling

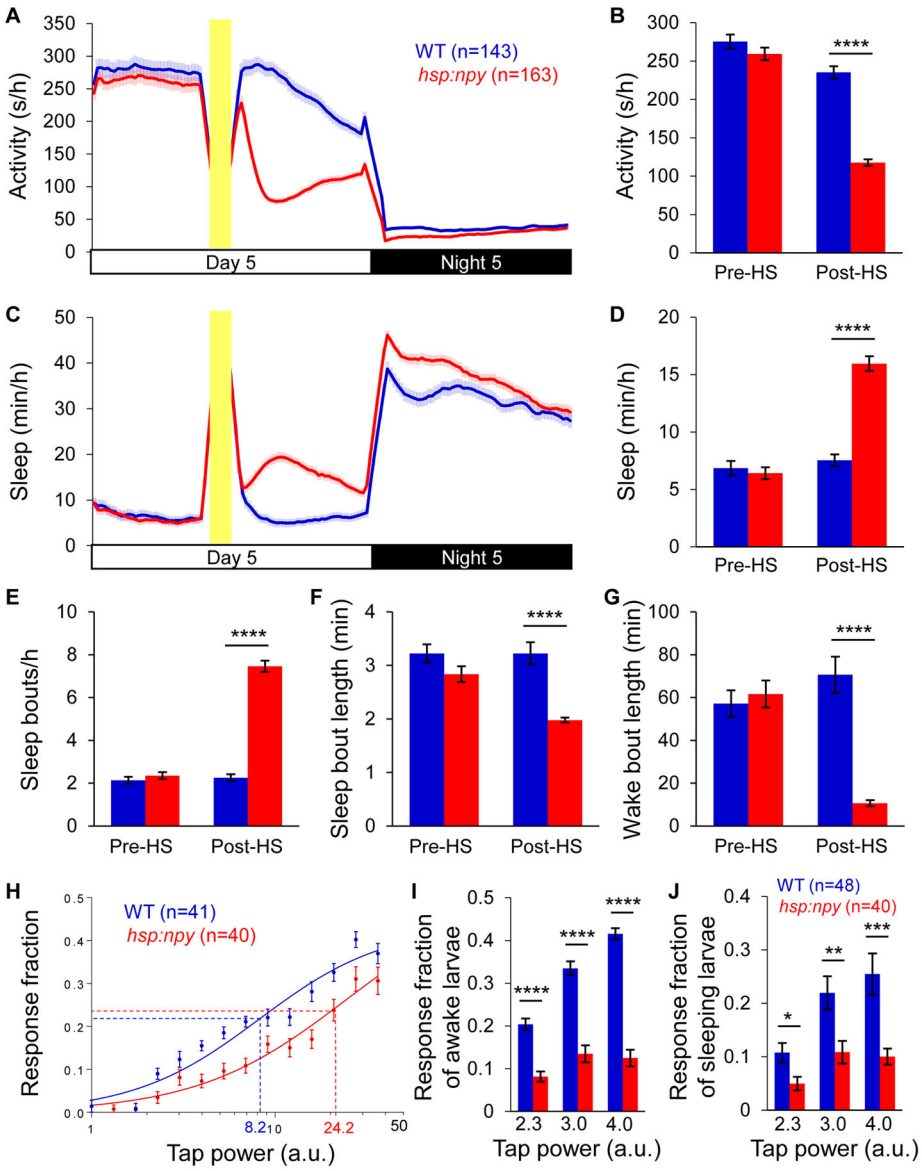

Figure 1. Overexpression of zebrafish NPY increases sleep and arousal threshold

(A–G) Overexpression of zebrafish NPY following a heat shock at 3 p.m. resulted in decreased locomotor activity (**A,B**) and increased sleep (**C,D**), due to more sleep bouts (**E**) and shorter sleep (**F**) and wake (**G**) bouts. Yellow bars indicate heat shock (HS). Pre-HS and Post-HS quantify data for day 5 before and after heat shock. Mean ± SEM from 4 experiments is shown. (**H**) Representative stimulus-response curve for *Tg(hsp:npy)* animals compared to WT siblings following heat shock. Data points represent mean ± SEM. Dashed lines mark ETP_{50} value for each genotype. *Tg(hsp:npy)* animals had an ETP_{50} value of 24.2 vs. 8.2 for WT siblings (293% increase, $P < 0.05$ by extra sum-of-squares F test). (**I,J**) Overexpression of NPY reduced the response of *Tg(hsp:npy)* animals to the stimulus compared to WT siblings during both awake and sleep states. Stimulus intensities of 2.3, 3.0 and 4.0 arbitrary units (a.u.) were tested. A dose-dependent response was observed for WT animals but not their *Tg(hsp:npy)* siblings. Bar graphs show mean ± SEM. n=number of

animals. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-tailed Student's t test. See also Figures S1, S2 and Movie S1.

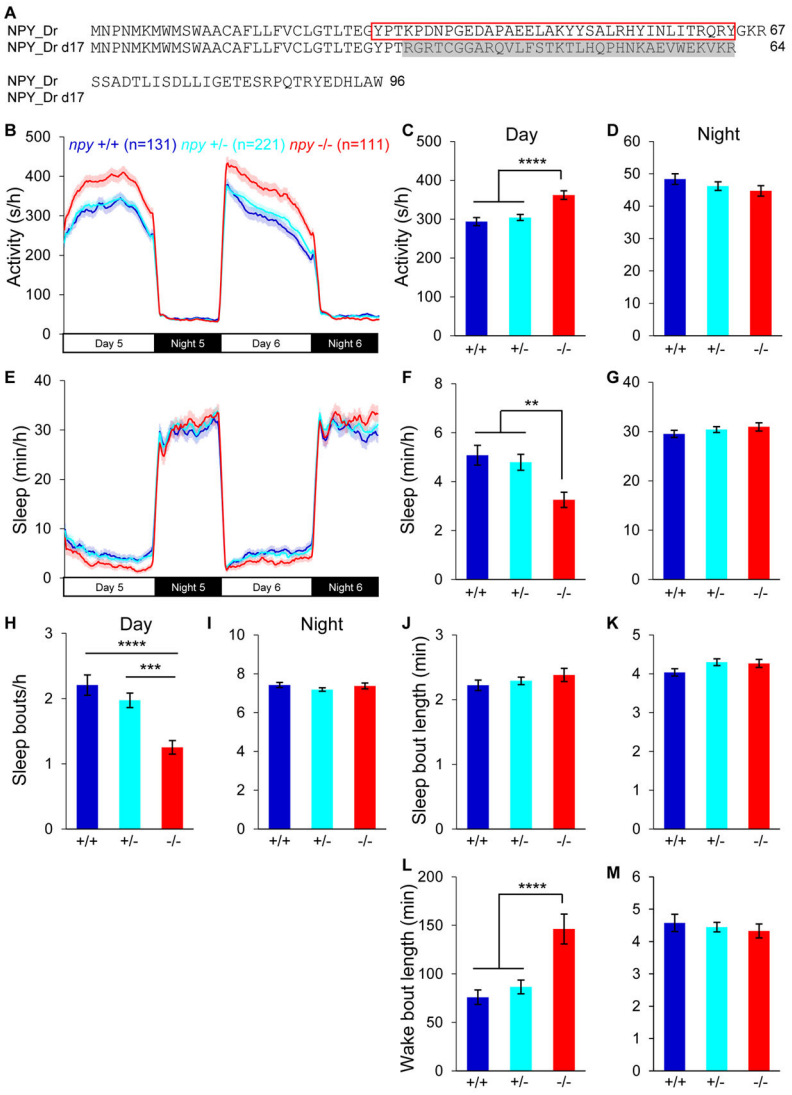

Figure 2. Loss of *npy* reduces daytime sleep

(**A**) Sequences of WT and mutant zebrafish NPY proteins. The mature peptide is indicated with a red box. Altered amino acids in the mutant are shaded grey. (**B–M**) *npy*−/− animals were more active (**B,C**), and slept less (**E,F**), than their *npy*+/+ and *npy*+/− siblings during the day, due to fewer sleep bouts (**H**) and longer wake bouts (**L**). Mean ± SEM from 7 experiments is shown. n=number of animals. ***P*<0.01; ****P*<0.001; *****P*<0.0001 by one-way ANOVA with Holm-Sidak test.

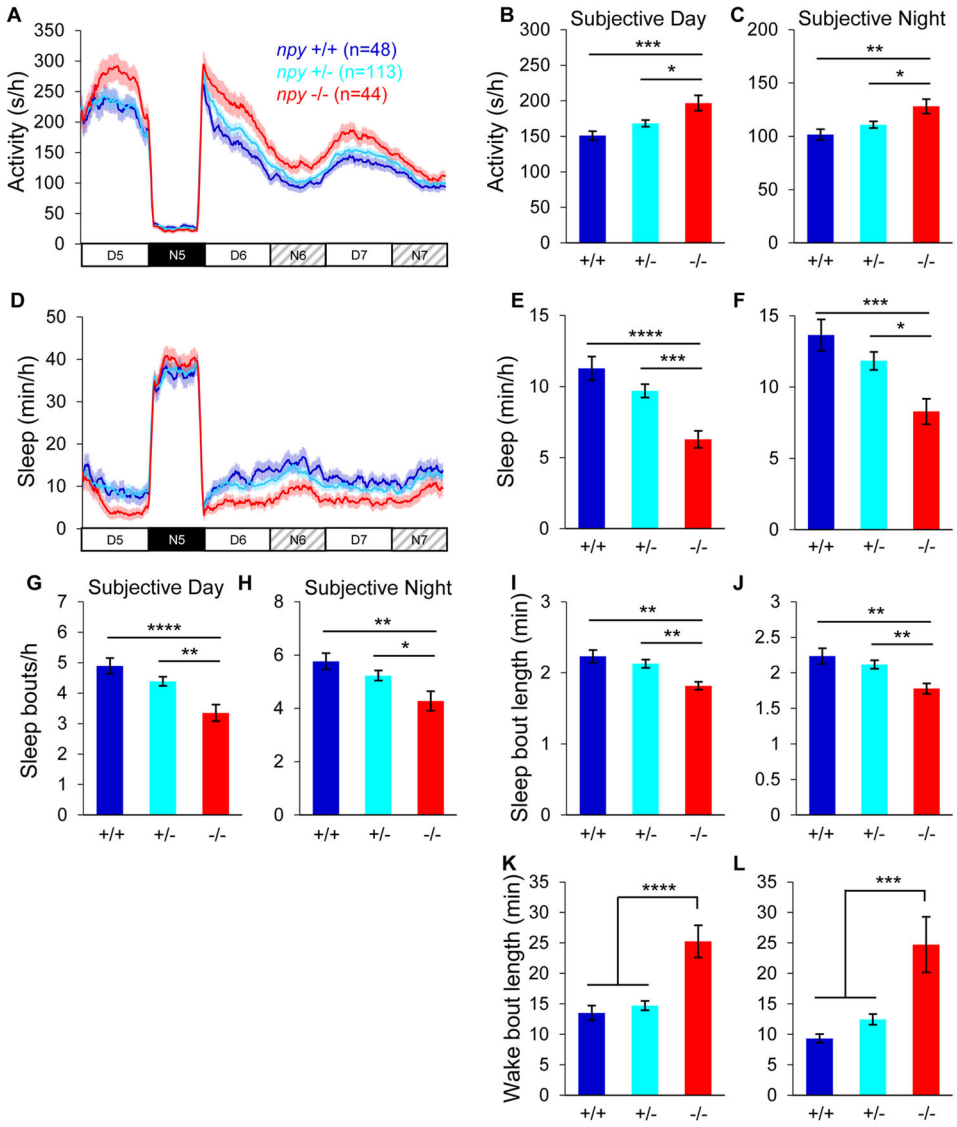

Figure 3. Entrained *npy* mutants sleep less in constant light

Larvae were entrained in 14:10 hour LD cycles for the first 4 days and nights of development, then behaviorally monitored for 24 hours in LD and then for 48 hours in LL. *npy*−/− animals were more active (**A–C**) and slept less (**D–F**) than their *npy*+/− and *npy*+/+ siblings during subjective day and night, due to fewer (**G,H**) and shorter (**I,J**) sleep bouts, and longer wake bouts (**K,L**). Mean ± SEM from 3 experiments is shown. n=number of animals. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 by one-way ANOVA with Holm-Sidak test. See also Figure S3.

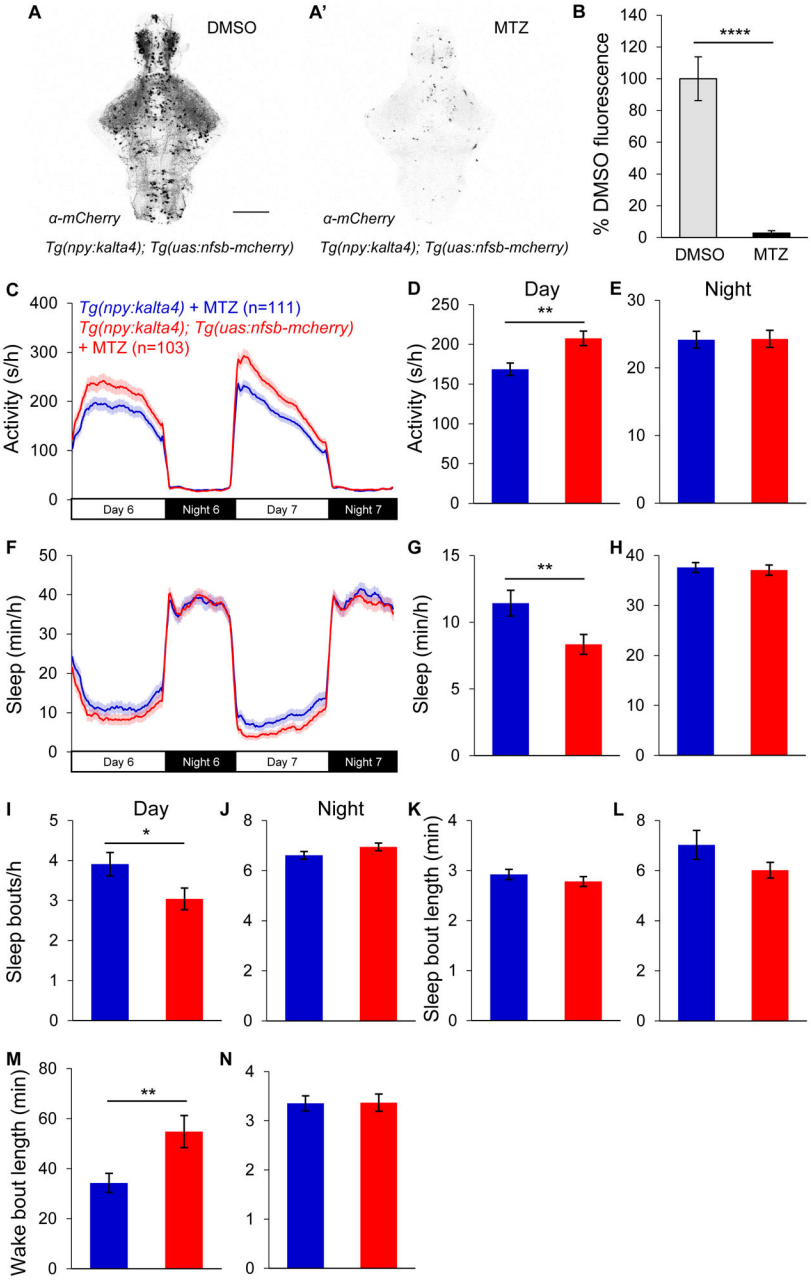

Figure 4. Loss of *npy*-expressing neurons reduces daytime sleep

(**A**) Ventral views of brains from 5 dpf *Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* animals stained with anti-DsRed antibody following treatment with DMSO (**A**) or 10 mM MTZ (**A′**), showing nearly complete loss of mCherry after MTZ treatment. (**B**) Mean ± SEM mCherry fluorescence intensity for *Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* animals treated with DMSO (n=4) or MTZ (n=4). (**C–N**) *Tg(npy:kalta4);Tg(uas:nfsb-mcherry)* animals treated with MTZ were more active (**C,D**) and slept less (**F,G**) than identically treated *Tg(npy:kalta4)* siblings during the day, due to fewer sleep bouts (**I**) and longer wake bouts (**M**). Mean ±

SEM from 3 experiments is shown. n=number of animals. *P<0.05; **P<0.01; ****P<0.0001 by two-tailed Student's t test. See also Figures S4, S5 and Table S1.

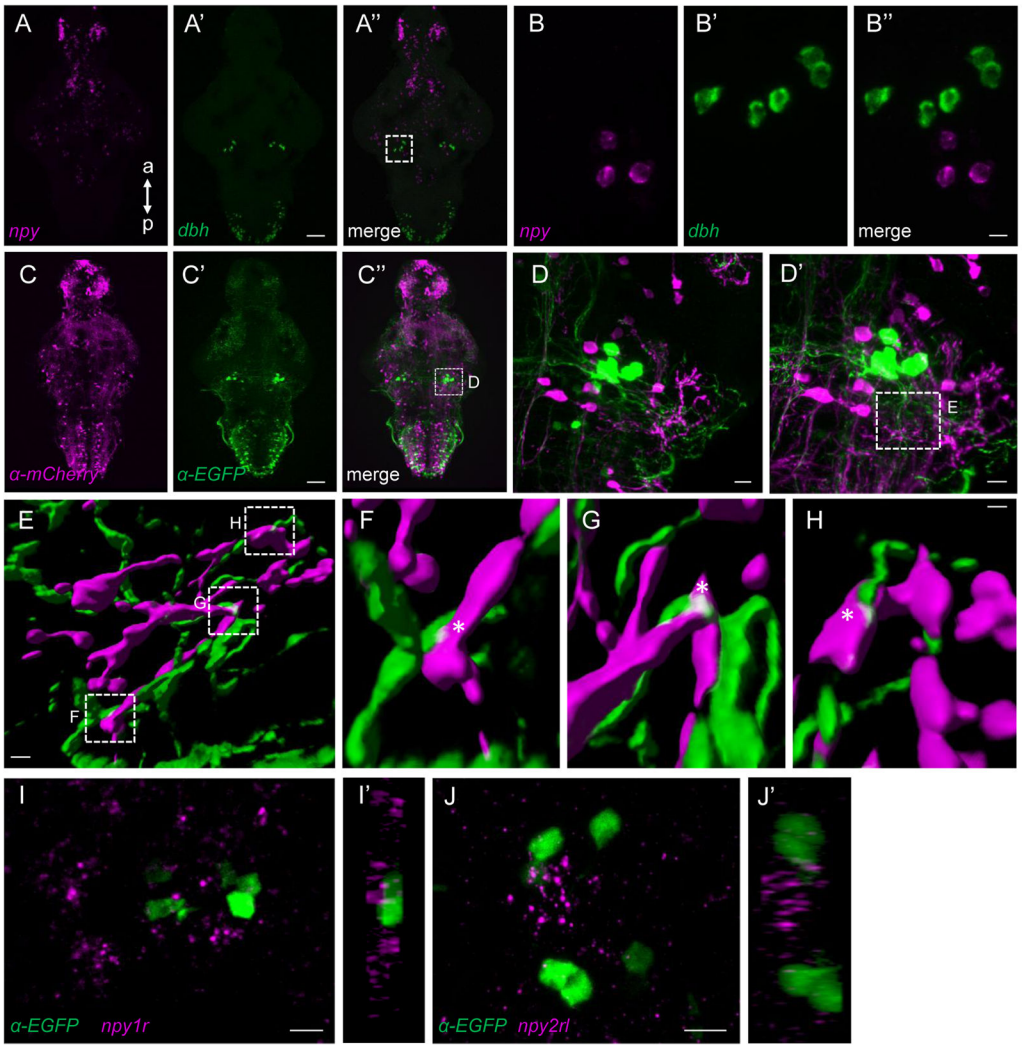

Figure 5. Evidence for anatomical interaction between hindbrain NPY neurons and the LC

(**A**) Double FISH using probes specific for *npy* and *dbh* show their close proximity in the LC. Boxed region in (**A″**) is magnified in a 50 μm thick maximum intensity projection in (**B**). (**C**) *Tg(npy:kalta4);Tg(uas:nfsb-mcherry);Tg(dbh:EGFP)* brains labeled using anti-DsRed and anti-EGFP antibodies. Boxed region in (**C″**) is magnified 25x in (**D**) and 63x in (**D′**). Maximum intensity projections 40 μm and 63 μm thick are shown in (**D**) and (**D′**). (**E**) Surface renderings of the boxed region in (**D′**). Boxed regions are magnified in (**F–H**). White asterisks show close proximity of NPY and LC neuron projections. (**I–J**) ISH using *npy1r*- and *npy2rl*-specific probes and immunostaining using an anti-EGFP antibody in *Tg(dbh:EGFP)* brains reveal close proximity of *npy1r* (**I**) and *npy2rl* (**J**) to *dbh*-expressing LC neurons. (**I′**) and (**J′**) show orthogonal views of the 24 μm and 25 μm thick maximum intensity projections shown in (**I**) and (**J**). a, anterior; p, posterior. Samples are 5 dpf brains. Scale bar: (**A–C**) 50 μm, (**B,D**) 10 μm, (**D′**) 7.5 μm, (**E**) 2.0 μm and (**F–H**) 0.5 μm. See also Movie S2.

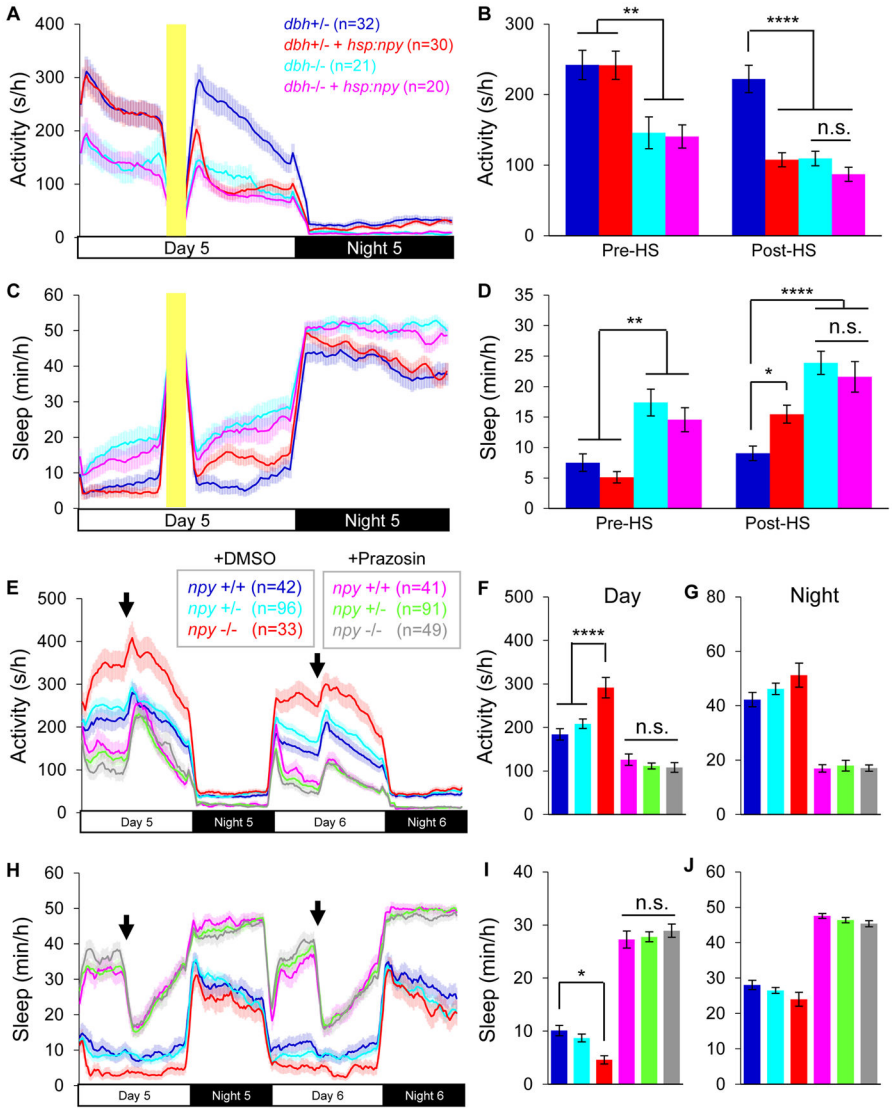

Figure 6. Functional evidence that NPY promotes sleep by inhibiting NE signaling

(**A–D**) *Tg(hsp:npy);dbh−/−* and *dbh−/−* animals were less active (**A,B**) and slept more (**C,D**) than *dbh+/−* siblings during the day before and after heat shock. *Tg(hsp:npy);dbh+/−* animals were less active and slept more than *dbh+/−* siblings during the day after heat shock. NPY overexpression in *Tg(hsp:npy);dbh−/−* animals did not further decrease locomotor activity or increase sleep compared to *dbh−/−* siblings. Yellow bars indicate heat shock (HS). Pre-HS and Post-HS quantify data on day 5 before and after heat shock. (**E–J**) *npy*+/+, *npy*+/− and *npy−/−* siblings were treated with either DMSO or prazosin. DMSO-treated *npy −/−* animals were more active (**E,F**) and slept less (**H,I**) than their DMSO-treated *npy*+/− and *npy+/+* siblings during the day. Prazosin decreased activity (**E,F**) and increased sleep (**H,I**) to a similar extent for *npy−/−*, *npy*+/− and *npy*+/+ siblings. Arrows indicate behavioral artifacts due to addition of water. Mean ± SEM for 2 (**A–D**) or 4 (**E–J**) experiments is shown. n=number of animals. n.s.=not significant, **P*<0.05; ***P*<0.01; *****P*<0.0001 by two-way ANOVA, with Holm-Sidak test. See also Figures S6, S7 and Table S2.

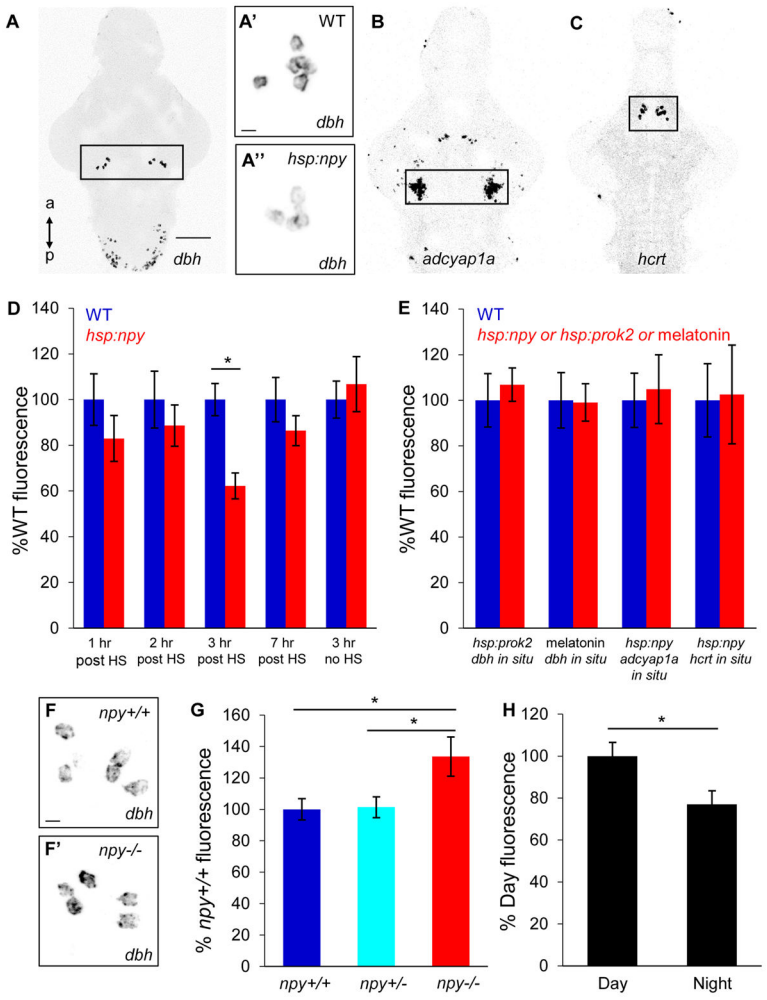

Figure 7. NPY signaling affects *dbh* mRNA level in the LC

(**A**) ISH showing *dbh* expression in the LC (boxed) and medulla oblongata. *dbh* mRNA levels were lower in *Tg(hsp:npy)* animals (**A″**) compared to WT siblings (**A′**) after heat shock. ISH using probes specific for *adcyap1a* (**B**) and *hcrt* (**C**). Boxed regions in (**A–C**) are quantified in (**D,E**). (**D**) *dbh* mRNA level in the LC is lower in *Tg(hsp:npy)* animals than WT siblings at 3 hours post-HS, but there is no significant difference at 1, 2, or 7 hours post-HS. (**E**) Overexpression of Prok2 or treatment with 20 μM melatonin had no effect on *dbh* expression. NPY overexpression did not affect *adcyap1a* or *hcrt* expression. (**F–F′**) *dbh* mRNA level in the LC was higher in *npy*−/− animals (**F′**) compared to *npy*+/+ siblings (**F**). (**G**) Quantification of *dbh* mRNA level in the LC of *npy*−/− animals and sibling controls. (**H**) *dbh* mRNA level in the LC of WT animals was lower at night than the day. Mean ± SEM fluorescence intensity from 8–12 brains for each condition is shown. **P*<0.05 by two-tailed Student's *t* test (**D,H**) or by one-way ANOVA with Holm-Sidak test (**G**). a, anterior; p, posterior. Samples are 5 dpf brains. Scale bar: (**A,B,C**) 100 μm; (**A′,A″,F,F′**) 10 μm.