A Zebrafish Genetic Screen Identifies Neuromedin U as a Regulator of Sleep/Wake States

Highlights

- A zebrafish genetic screen identifies genes that regulate sleep and arousal

- Nmu overexpression inhibits sleep whereas *nmu* mutants are hypoactive

- Nmu-induced phenotypes require Nmu receptor 2 but not the glucocorticoid receptor

- Nmu-induced arousal activates brainstem Crh neurons and requires Crh signaling

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In Brief

Chiu et al. perform a genetic screen in zebrafish and identify Nmu as a regulator of sleep/wake behaviors. They show that Nmu overexpression activates brainstem Crh neurons and that Nmu-induced arousal requires Crh signaling, thus identifying a novel vertebrate arousal circuit.

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Article

A Zebrafish Genetic Screen Identifies Neuromedin U as a Regulator of Sleep/Wake States

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SUMMARY

Neuromodulation of arousal states ensures that an animal appropriately responds to its environment and engages in behaviors necessary for survival. However, the molecular and circuit properties underlying neuromodulation of arousal states such as sleep and wakefulness remain unclear. To tackle this challenge in a systematic and unbiased manner, we performed a genetic overexpression screen to identify genes that affect larval zebrafish arousal. We found that the neuropeptide neuromedin U (Nmu) promotes hyperactivity and inhibits sleep in zebrafish larvae, whereas *nmu* mutant animals are hypoactive. We show that Nmu-induced arousal requires Nmu receptor 2 and signaling via corticotropin releasing hormone (Crh) receptor 1. In contrast to previously proposed models, we find that Nmu does not promote arousal via the hypothalamic-pituitary-adrenal axis, but rather probably acts via brainstem *crh*-expressing neurons. These results reveal an unexpected functional and anatomical interface between the Nmu system and brainstem arousal systems that represents a novel wake-promoting pathway.
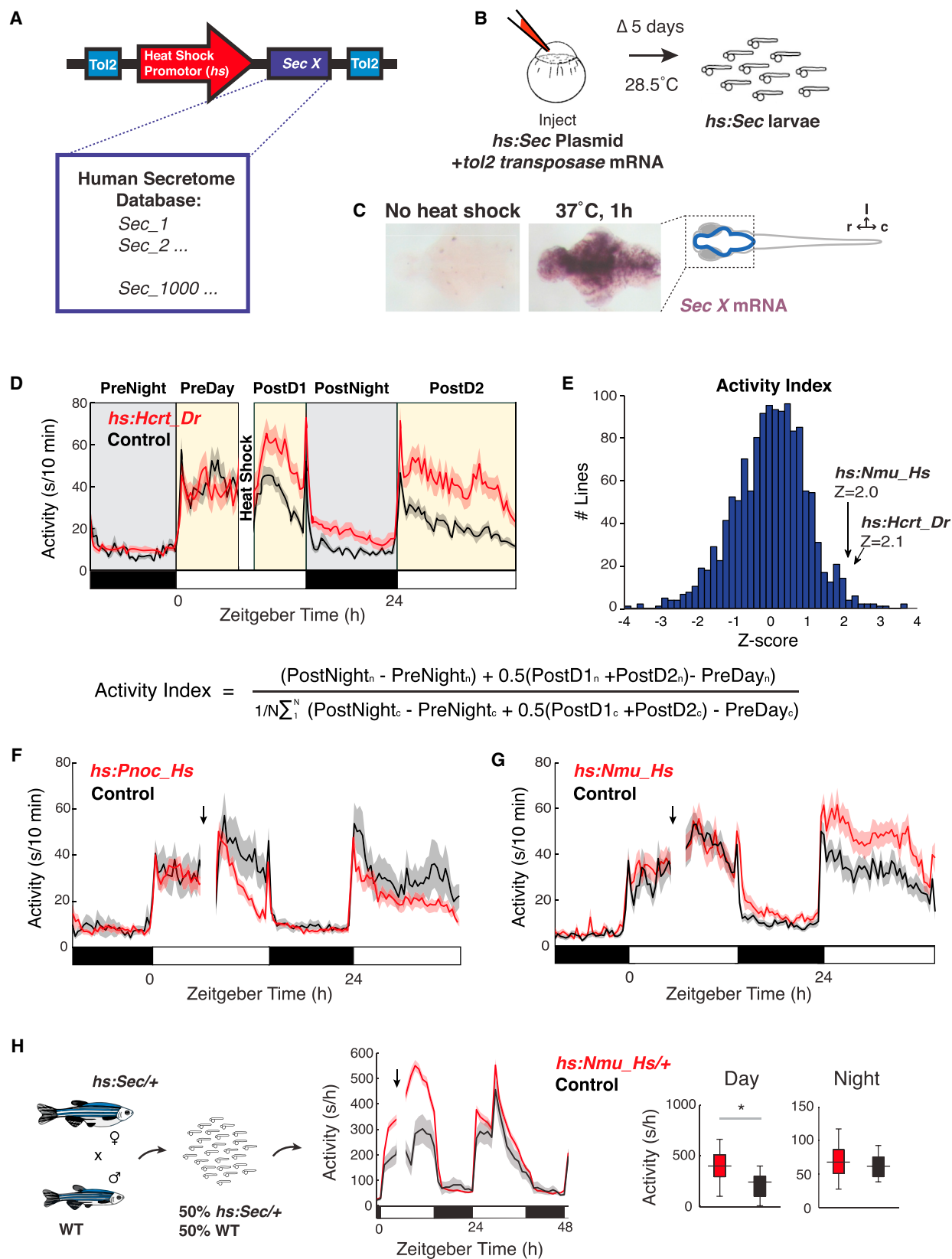
INTRODUCTION

A major challenge in neuroscience is to understand the molecular and neural circuitry that regulates arousal states such as sleep and wakefulness, which are fundamental to animal behavior and function. While decades of research have uncovered some of the molecular, anatomical, and functional properties of the brain systems that regulate sleep and wakefulness, the overwhelming evidence indicates that there are many parallel systems regulating vertebrate arousal states, with many players remaining to be discovered.

Large-scale screening offers the potential to discover novel mechanisms of arousal, including sleep/wake regulation, but the challenge of performing behavioral genetic screens using mammalian model organisms is daunting. While genetic screens for sleep/wake regulators have been successful in *Drosophila* (Cirelli et al., 2005; Koh et al., 2008; Pfeiffenberger and Allada, 2012; Rogulja and Young, 2012; Stavropoulos and Young, 2011), flies lack mammalian sleep regulators like orexin/hypocretin (Hcrt), and some of the genes identified in fly screens lack clear vertebrate orthologs. In contrast to mammals, zebrafish (*Danio rerio*) are well suited for large-scale screens. Indeed, two large-scale forward genetic screens for developmental and simple behavioral phenotypes stimulated the widespread adoption of zebrafish as a model system (Driever et al., 1996; Haffter et al., 1996). Though fruitful, traditional F2 screening approaches are associated with a high cost because they require the generation of large collections of mutant lines and considerable challenges in identifying the causative genetic lesions. F1 screens bypass this requirement and therefore offer the advantage of efficiency in time, expense, and labor.

Another obstacle to understanding the regulation of sleep/wake states is the challenge of functionally and anatomically dissecting complex mammalian arousal systems. One source of complexity is the presence of multiple neurochemical and anatomical systems with overlapping or interlinked functions in arousal. For example, some constitutive loss-of-function models that disrupt well-studied arousal pathways, such as those mediated by adenosine, histamine, and noradrenaline, yield only

Activity Index $=$ $\dfrac{(\text{PostNight}_n - \text{PreNight}_n) + 0.5(\text{PostD1}_n + \text{PostD2}_n) - \text{PreDay}_n)}{1/N \sum_{1}^{N} (\text{PostNight}_c - \text{PreNight}_c + 0.5(\text{PostD1}_c + \text{PostD2}_c) - \text{PreDay}_c)}$

(legend on next page)

subtle or no sleep phenotypes in rodents (Huang et al., 2007; Hunsley and Palmiter, 2003; Thakkar, 2011). In contrast, acute pharmacological manipulations of these pathways have profound effects on sleep/wake behaviors (Huang et al., 2007; Hunsley and Palmiter, 2003; Thakkar, 2011), suggesting that developmental compensatory mechanisms may mask phenotypes in constitutive loss-of-function models. Thus, in general, the redundancy of arousal pathways makes inducible, gain-of-function manipulations more suitable for identifying mechanisms involved in arousal.

To complement the existing repertoire of screening and circuit characterization approaches, we developed a screening and follow-up approach that leverages several features of the zebrafish system. We designed and implemented an injection-based, inducible gene overexpression screening methodology to identify genes that regulate vertebrate sleep/wake behavior. This approach offers the power of inducible, gain-of-function approaches combined with the efficiency of F1 screens. To our knowledge, this is the largest-scale genetic screen for sleep/wake regulators in a vertebrate animal. In this screen, we identified neuromedin U (Nmu) as a neuropeptide regulator of zebrafish sleep/wake behavior. Using a combination of pharmacology and genetics, we also provide a mechanistic basis for Nmu-mediated arousal in zebrafish that shares some features of Nmu signaling in mammals. In mammals, Nmu has been shown to promote locomotor activity, which was hypothesized to act via the hypothalamic-pituitary-adrenal (HPA) axis (Hanada et al., 2001). In contrast to this model, we find that Nmu overexpression-induced phenotypes do not require HPA axis signaling. Instead, we uncovered an unexpected and previously unexamined link between the Nmu system and brainstem arousal circuits. Our data implicate a new circuit in the regulation of vertebrate arousal and, more broadly, validates our genetic screen design.

RESULTS

A Zebrafish Inducible Genetic Overexpression Screen for Sleep Regulators

Our genetic screen features four design components (Figures 1A–1C). First, we leveraged the hORFeome 3.1 collection (http://horfdb.dfci.harvard.edu), a publicly available set of cloned human open reading frames (ORFs, i.e., potential protein-coding regions of DNA), to conduct the screen on a large scale. We used human genes because a resource of zebrafish

ORFs was not available, and we reasoned that most human peptides would be functional in zebrafish due to high sequence conservation. We cross-referenced the hORFeome 3.1 collection with the LOCATE database (http://locate.imb.uq.edu.au), which annotates 4,418 human ORFs that are known or predicted to encode secreted proteins (Secretome). The hORFeome 3.1 contains 1,632 of these ORFs. Thus, we cloned over one-third (1,632 ORFs in hORFeome 3.1/4,418 ORFs in the human Secretome = 37%) of the human Secretome for testing in zebrafish. We focused on secreted peptides because several known sleep regulators are secreted neuropeptides and because secreted peptides act non-cell autonomously, a technical advantage in that transgenes do not need to be overexpressed in all cells for secreted peptides to reach their targets. Second, we used an inducible overexpression transgene vector (hs:Sec, or Heat-Shock Secretome Gene) to avoid effects on development. Overexpression of a given Secretome candidate gene was achieved by incubating zebrafish larvae at 37°C for 1 hr (Figure 1C). Third, we used a transgene injection approach for the primary screen. By directly testing larvae injected with a hs:Sec transgene, we circumvented the considerable labor, cost, and time required to test stable transgenic lines. This approach is possible using the Tol2 transposase (Asakawa and Kawakami, 2009), which efficiently integrates injected plasmids into the genome of injected embryos (Figures 1B and 1C). We performed a proof-of-principle experiment with a plasmid in which the heat shock promoter regulates expression of the zebrafish Hcrt ortholog (hs:Hcrt_Dr) (Figure 1D), which confirmed that our injection-based approach achieves the same inducible hyperactivity as the previously published Tg(hs:Hcrt_Dr) stable transgenic line (Prober et al., 2006). Fourth, we employed a high-throughput locomotor activity assay (Prober et al., 2006) to identify genes that, when overexpressed, produced a quantifiable sleep/wake phenotype. Satisfying the behavioral criteria that distinguish sleep from inactivity in animals (Allada and Siegel, 2008), zebrafish larvae exhibit a diurnal pattern of activity and rest (Cahill et al., 1998; Zhdanova et al., 2001), increased rest following rest deprivation (Zhdanova et al., 2001), and an increased arousal threshold following 1 or more minutes of rest (Elbaz et al., 2012; Prober et al., 2006). Thus, 1 or more minutes of rest can be considered a sleep-like state in zebrafish larvae. Several groups have also demonstrated anatomical, genetic, and pharmacological conservation of sleep between zebrafish and mammals, establishing zebrafish as a simple

Figure 1. A Behavioral Genetic Overexpression Screen
(A) The Heat Shock Secretome (hs:Sec) overexpression transgenesis cassette.
(B) In the primary screen, single cell zebrafish embryos were co-injected with the hs:Sec plasmid and tol2 transposase mRNA. Embryos were raised to 5 days post-fertilization (dpf) at 28.5°C, a non-permissive temperature for the heat shock promoter.
(C) A 1 hr, 37°C heat shock (but not the 28.5°C control) treatment induces expression of a Secretome gene in hs:Sec/+ larvae, as visualized by in situ hybridization (purple stain) in dissected brains.
(D) Average activity traces for hs:Hcrt_Dr-injected (n = 32) and control injected (n = 16) larvae.
(E) Histogram of all activity indices from the screen, normalized as standard deviations from the mean (Z score).
(F) Average activity traces of controls and hs:Pnoc_Hs, a previously identified regulator of sleep/wake behavior in zebrafish.
(G) Average activity traces of controls and hs:Nmu_Hs, a newly identified regulator of sleep/wake behavior in zebrafish.
(H) Candidate genes were retested as stable transgenic lines. hs:Nmu_Hs/+ larvae generated from a stable transgenic line exhibited increased activity compared to their WT siblings. Line plot values are represented as mean ± SEM. White and black boxes on the x axis of the line plots denote daytime light periods and nighttime dark periods, respectively. Arrows indicate time of heat shock (F–H). *p < 0.05, Mann-Whitney U test.

vertebrate model for sleep research (reviewed in Chiu and Prober, 2013; Zhdanova, 2011).

In the primary screen, we performed 1,432 experiments that tested 1,286 unique ORFs covering 1,126 unique genes (Table S1). We therefore screened 29% (1,286/4,418) of the human Secretome, which is the largest screen to date for genes that affect vertebrate sleep/wake patterns. For each ORF, we tested 32 injected embryos in the sleep/wake assay along with 16 matched controls that were injected with either *hs:EGFP* or an empty *hs* vector. To assess the effects of each ORF on behavior, we divided each behavioral trace into pre- and post-heat shock epochs for each day and night (Figure 1D) and calculated alterations in sleep amount and structure (e.g., number and length of sleep bouts, sleep latency at night), as well as changes in activity levels (Figure S1; Table S2). One such measure is the Activity Index, an assessment of the change in activity due to overexpression of an ORF normalized to the set of controls and internally controlled to the pre-HS epoch (Figure 1D). The collection of Activity Indices is normally distributed (Figure 1E) and identifies ORFs that up- or downregulate activity when overexpressed. We calculated similar measures for effects on several other parameters, including total sleep, sleep bout number and length, sleep latency, and day activity levels (Figures S1A–S1F). Based on these initial assessments of the primary screen, we selected 60 ORFs to confirm in stable transgenic lines (see Supplemental Experimental Procedures): 31/60 (52%) were chosen from the top ranks of the Activity Index (e.g., those that most strongly decreased or increased activity; Figure 1E), 13/60 (22%) were based on the Night Sleep Index (Figure S1B), and the remaining 16/60 (26%) were based on strong single measure effects, for example, alterations in sleep structure like the number or length of sleep bouts, or strong day versus night effects (Figures S1C–S1F). These measures are capable of identifying regulators of zebrafish sleep and activity, including hypocretin (Hcrt), a previously described promoter of activity (Figures 1D and 1E, Z score Activity Index = 2.1) (Prober et al., 2006), and prepronociceptin (Pnoc), a previously described repressor of activity (Figures 1F and S1F, Z score Activity Index = −1.3; Z Score Post-HS Day Activity, −2.0) (Woods et al., 2014). Table S2 provides summary statistics for these sleep/wake parameters. Of the 60 peptides overexpressed in stable transgenic lines, 10 (17%) gave reproducible phenotypes as expected from the primary screen and another two lines gave unexpected phenotypes (Table S3). These will be described in more detail elsewhere.

Identification of Neuromedin U as a Neuropeptidergic Regulator of Sleep/Wake Behavior in Zebrafish

One strong enhancer of post-HS locomotor activity was Nmu (Figures 1E and 1G; activity index Z score = 2.0). Overexpression of human Nmu (*hs:Nmu_Hs*) significantly increased locomotor activity compared to pre-heat shock and to EGFP overexpressing controls (Figure 1G). Overexpression of human Nmu using a stable line generated from the same transgene also significantly increased locomotor activity, although the temporal dynamics of this phenotype was different from that observed in the screen, probably due to leaky *nmu* expression by the heat shock promoter in this stable line (Figure 1H).
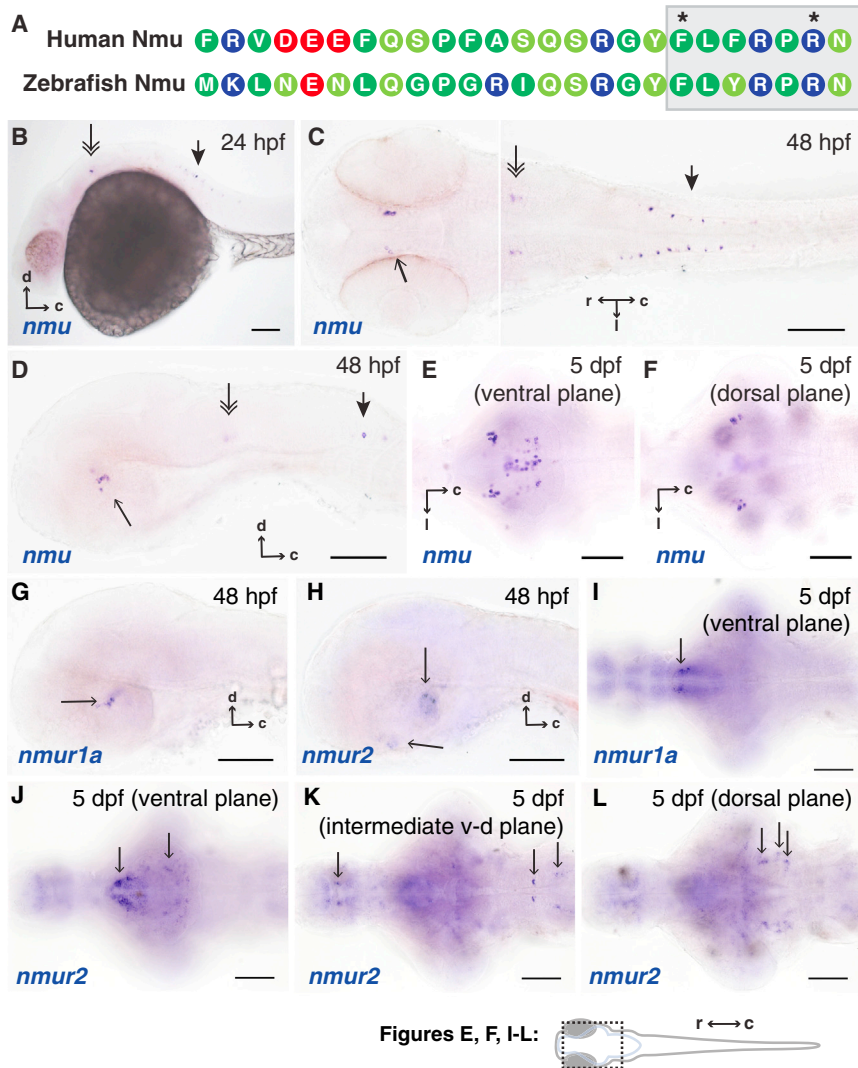
To determine whether the mammalian Nmu system is conserved in zebrafish, we first cloned the zebrafish ortholog. The zebrafish genome contains a single *nmu* ortholog that encodes a predicted 25 amino acid-length mature peptide that is 56% identical to human mature peptide Nmu-25 (Figures 2A, S2A, and S2B). Based on structure-function studies of vertebrate Nmu, the C-terminal heptapeptide sequence is known to contain the critical region for biological activity (Hashimoto et al., 1991; Sakura et al., 1991). There is significant conservation of this region, as zebrafish Nmu retains six of seven mammalian Nmu C-terminal amino acids, including a phenylalanine (F) residue that is important for activity (Kurosawa et al., 1996) and an arginine (R) residue that is indispensable for receptor binding and activation (Sakura et al., 2000) (Figures 2A and S2B).

Using in situ hybridization (ISH), we found that zebrafish *nmu* is expressed in discrete nuclei within the CNS of embryonic and larval zebrafish. At 24 hr post-fertilization (hpf), a single bilateral cell cluster occupies the caudal brainstem and an additional bilateral row of neurons is present in the spinal cord (Figure 2B). These cell populations persist at 48 hpf, and additionally a bilateral hypothalamic population appears at this stage (Figures 2C and 2D). At 120 hpf, additional cells differentiate in the hypothalamus (Figures 2E and 2F; Movie S1). This pattern of expression accords with *nmu* expression in the adult mammalian CNS in hypothalamus, brainstem, and spinal cord (Austin et al., 1994; Fujii et al., 2000; Graham et al., 2003; Howard et al., 2000; Ivanov et al., 2002; Nogueiras et al., 2006; Szekeres et al., 2000). We did not observe peripheral expression of *nmu* in the gastrointestinal or genitourinary tracts, or in the skin, as has been reported in mammals.

Next we examined the functional role of zebrafish Nmu. Most studies of Nmu in mammals have focused on its role in regulating appetite, but several studies have also shown that Nmu can induce short-term increases in locomotor activity or grooming following acute, central administration of Nmu peptide (Howard et al., 2000; Nakazato et al., 2000; Novak et al., 2006, 2007; Peier et al., 2009; Semjonous et al., 2009; Wren et al., 2002). Thus, we first asked whether overexpression of the zebrafish Nmu ortholog in zebrafish produces a similar increased locomotor activity phenotype. We found that heat shock-induced overexpression of the zebrafish Nmu ortholog using a stable transgenic line (*Tg(hs:Nmu_Dr)*) (Figure S3) recapitulated the increased locomotor activity phenotype induced by *Tg(hs:Nmu_Hs)* in zebrafish, causing a large increase in wake activity (i.e., amount of locomotor activity while awake; Figures 3A and 3A′). Thus, overexpression of Nmu in zebrafish and central administration of Nmu peptide in rodents both promote locomotor activity.

We further characterized the functional role of zebrafish Nmu by employing several additional assays of arousal. First, we characterized the effects of Nmu overexpression on spontaneous sleep/wake architecture. We found that sleep in Nmu-overexpressing larvae is dramatically reduced during both day and night and is almost completely abolished during the night following heat shock (Figures 3B and 3B′). Further analysis of the effects of Nmu overexpression on sleep structure at night revealed an increase in sleep latency (Figure 3C), a decrease in the frequency of sleep bouts (Figure 3D), a decrease in sleep bout length (Figure 3E), and a reciprocal increase in wake bout length

Figure 2. The Sequence of Nmu and Expression of *nmu* and *nmu receptors* Are Conserved in Zebrafish

(A) The zebrafish predicted Nmu peptide sequence is well conserved with the human Nmu mature peptide. The C-terminal heptapeptide and critical residues are indicated by the shaded box and asterisks. Amino acids are color coded by type (after Malendowicz et al., 2012): blue, basic; red, acidic; green, non-polar; light green, polar.

(B–F) Endogenous expression of *nmu* in the hypothalamus (single arrow), brainstem (double arrow), and spinal cord (arrowhead) of 24–120 hpf zebrafish.

(G and H) Lateral views of *nmur1a* and *nmur2* expression at 48 hpf.

(I) *nmur1a* expression in 5 dpf larvae is restricted to the rostro-ventral hypothalamus.

(J–L) Widespread but discrete expression of *nmur2* in 5 dpf zebrafish brain, starting from a ventral focal plane (J) and ending in a more dorsal focal plane (L). Note that a prominent population of cells in the hypothalamus (left/rostral arrow in J) does not localize to the zebrafish preoptic nucleus, which is not visible in this focal plane. Also note several discrete clusters of cells in the hindbrain (arrows in L), which are located in the vicinity of brainstem arousal systems such as the locus coeruleus. In (G)–(L), arrows distinguish cellular labeling from diffuse background staining. Scale bars = 100 μm.

behavioral paradigm in which larval zebrafish raised and tested in LD were exposed to brief, 1 min light pulses once every 2 hr throughout the dark phase (Figure 3G). We characterized the responses of wild-type (WT) larvae to the light pulse trials and found that, on average, larvae respond in a biphasic manner (Figure 3G). The first behavioral epoch, which we call the "stimulus-on" response, occurs within a few seconds of the stimulus onset. The second behavioral epoch, which we call the "post-stimulus" response, is initiated several minutes after stimulus offset. To quantify the behavioral responses during both of these epochs, we measured the peak amplitude and total activity, as well as decay kinetics by fitting a single exponential curve to each response epoch (see Figures 3G and 3H and Supplemental Experimental Procedures for details). Using these measures, we found that the activity of WT larvae during the stimulus-on epoch decays with a time constant of $\tau = 55.2 \pm 10.4$ s (mean ± SD, n = 29, Figures 3G and 3K), whereas the post-stimulus activity persists much longer and decays with a time constant of $\tau = 10.4 \pm 1.9$ min (mean ± SD, n = 29, Figures 3G and 3N). We found that Nmu overexpression modulates the stimulus-on response by shortening and reducing the total activity (Figures 3J–3L). Conversely, Nmu overexpressing larvae exhibit a larger, more prolonged response during the following post-stimulus epoch (Figures 3M–3O). Consistent with the effects of Nmu overexpression in our sleep/wake assay, we also observed an increase in baseline activity prior to stimulus onset in Nmu-overexpressing
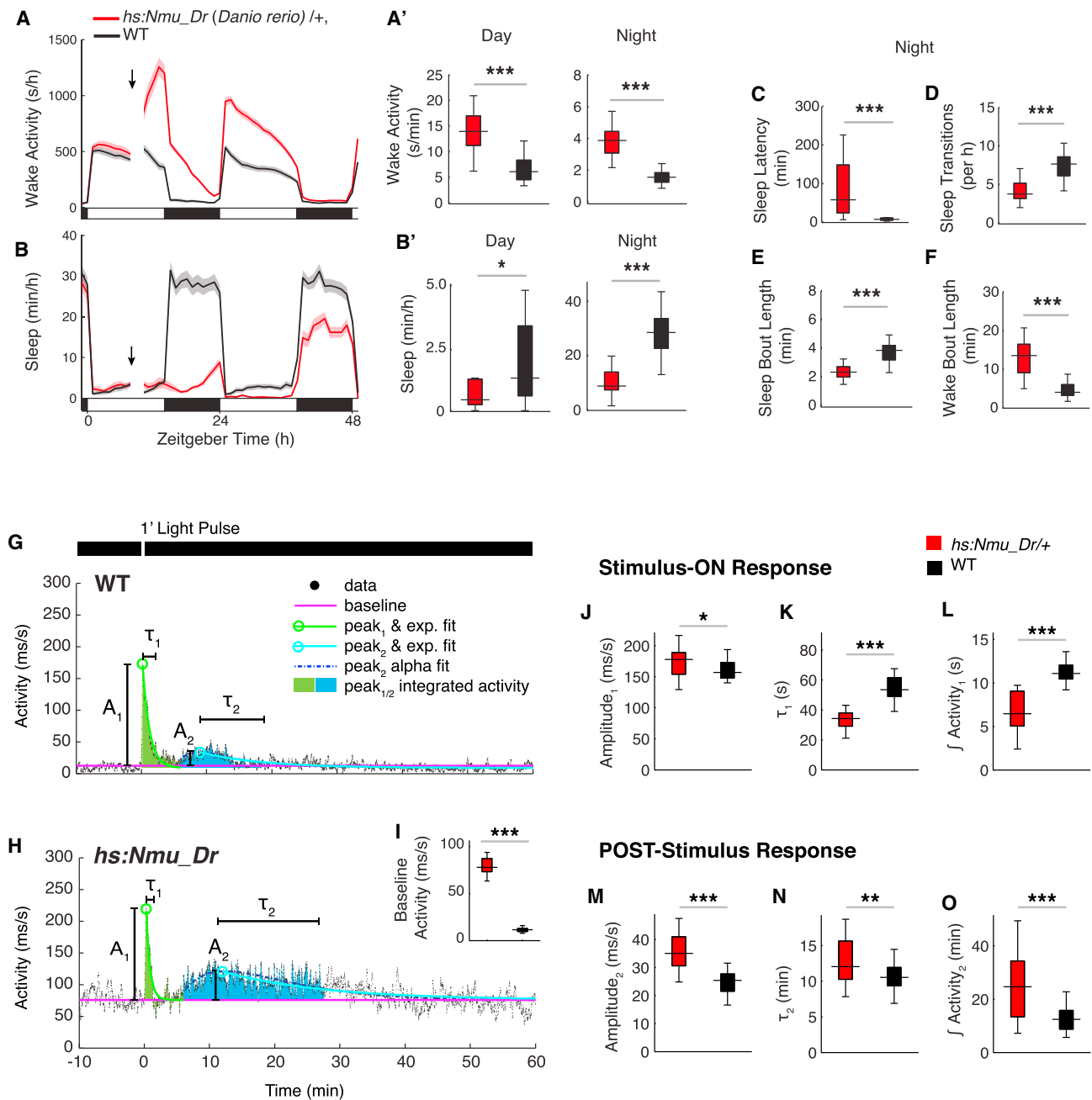
(Figure 3F). Thus, the overall effect of Nmu overexpression on sleep/wake architecture is to cause hyperactivity, reduce total sleep time, and disrupt the initiation and maintenance of sleep, resulting in a severe insomnia-like phenotype.

Next, we asked whether Nmu-induced phenotypes require external circadian entrainment signals (zeitgebers) such as light. First, we tested larvae that were entrained to 14:10 hr light/dark (LD) conditions and then shifted into constant light (LL) or constant dark (DD) conditions. We observed that Nmu-induced phenotypes persist in LL or DD after LD entrainment (Figures S4A–S4D′), indicating that external entraining light cues are not required for Nmu-induced phenotypes. We next tested larvae that had been raised without circadian entrainment (i.e., raised and tested in LL) and found that Nmu-induced phenotypes do not require rhythmic behavior (Figures S4E and S4F′). Together, these results suggest that neither external nor internal circadian cues are required for Nmu overexpression to modulate behavior.

We also asked whether Nmu can modulate arousal responses to acute external stimuli in addition to modulating spontaneous baseline activity. To test this hypothesis, we developed a simple

Figure 3. Nmu Overexpression Modulates Spontaneous Activity, Sleep, and Stimulus-Evoked Arousal in Zebrafish

(A–F) Overexpression of zebrafish Nmu (*hs:Nmu_Dr/+*, n = 44) induces hyperactivity and suppresses sleep compared to WT siblings (WT, n = 43). (A and B) Line plots of mean ± SEM show a 48 hr recording of 6 dpf larvae raised and tested on a 14:10 hr light:dark cycle (white and black bars on x axis, respectively). Arrows indicate time of heat shock. Box plots (A′ and B′) quantify data from post-heat shock day or night periods. (C–F) Quantification of additional sleep parameters from the same dataset. Night sleep architecture is altered in *hs:Nmu_Dr/+* zebrafish by increased latency to first sleep (C), fewer (D) and shorter (E) sleep bouts, and longer wake bouts (F).

(G–O) A stimulus-evoked arousal assay reveals two epochs of behavioral arousal that are differentially modulated by Nmu overexpression. Measurement of baseline, peak amplitude (ypeak – baseline), decay kinetics (τ, single exponential fit), and total activity (shaded area) of each response phase are shown for representative WT (G) and *hs:Nmu_Dr/+* samples (H) during the night following heat shock. (I–O) Box plots show quantification of baseline, stimulus-on and post-stimulus response parameters for n = 29 samples per genotype. Each sample represents a 30-trial average response generated from the full sets of 870 trials pooled across individuals of each genotype. In all box plots (A′–F) and (I–O), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Mann-Whitney U test.

larvae (Figure 3I). Taken together, our studies of zebrafish locomotor activity under various stimulus conditions show that Nmu modulates arousal by increasing spontaneous activity and secondary stimulus-evoked arousal states and, paradoxically, Nmu modulates acute arousal responses by suppressing activity.

nmu Is Required for Normal Arousal Levels and Nmu Overexpression-Induced Arousal Requires nmur2

To determine whether *nmu* is required for normal arousal levels and to test the functional requirement of each *nmu receptor* (*nmur*) ortholog in mediating the Nmu overexpression phenotype, we generated zebrafish that contain a predicted null mutation in *nmu* and in each *nmu receptor* using zinc finger nuclease and TAL effector nuclease technologies (Figures S2C, S2D, and S2F). The zebrafish genome contains two zebrafish Nmur1 orthologs, designated Nmur1a and Nmur1b, that are 52% and 49% identical to human Nmur1 (Figures S2C–S2E and S2H), and a single ortholog of Nmur2 that is 44% identical to human Nmur2 (Figures S2F–S2H). Using ISH, we observed that *nmur2* is primarily expressed in discrete regions in the zebrafish brain, including cell clusters in the brainstem, hypothalamus, and forebrain (Figures 2H and 2J-2L; Movie S2). We detected more restricted expression of *nmur1a* in a discrete cluster of rostroventral hypothalamic cells (Figures 2G and 2I), and we detected no *nmur1b* transcript in zebrafish up to 120 hpf. Thus, like mammals, zebrafish *nmur2* expression in the CNS is more widespread compared to *nmur1* (reviewed by Brighton et al., 2004).

Consistent with the Nmu overexpression phenotype, we found that *nmu*−/− larvae were less active during the day (Figures 4A and 4A′). *nmu*−/− adults were also less active (Figures 4C and 4C′), with the largest effect just prior to and following the onset of light in the morning (Figures 4D and 4D′). Larvae lacking individual Nmu receptors exhibited largely normal sleep/wake behaviors (Figure S5A–S5F). However, *nmur2*−/− adults exhibited a phenotype similar to that of *nmu*−/− adults (Figures S5G and S5H), suggesting that either redundant mechanisms or maternal contribution may rescue the *nmur2*−/− phenotype in larvae but not adults. Furthermore, while the Nmu overexpression phenotype was unaffected in *nmur1a* and *nmur1b* mutants (Figures S5A–S5D), it was abolished in *nmur2* homozygous mutant larvae (Figure 5). These results suggest that Nmu and Nmur2 are required for normal arousal levels and that Nmu overexpression promotes arousal via Nmur2.

In addition to its requirement for normal arousal levels, we also observed that *nmu*−/− adult fish were smaller and weighed less than their heterozygous mutant and WT siblings (Figure S6). Surprisingly, we observed a similar phenotype in *nmur1a*−/− adults, but not in *nmur1b*−/− or *nmur2*−/− adults (Figure S6). No size differences were observed in any of the mutants at the larval stage. These observations suggest that different Nmu receptors have different functional roles, with Nmur2 required for Nmu-dependent arousal and Nmur1a required for Nmu-dependent growth in adult fish.

Nmu Overexpression Phenotype Does Not Require the Glucocorticoid Receptor

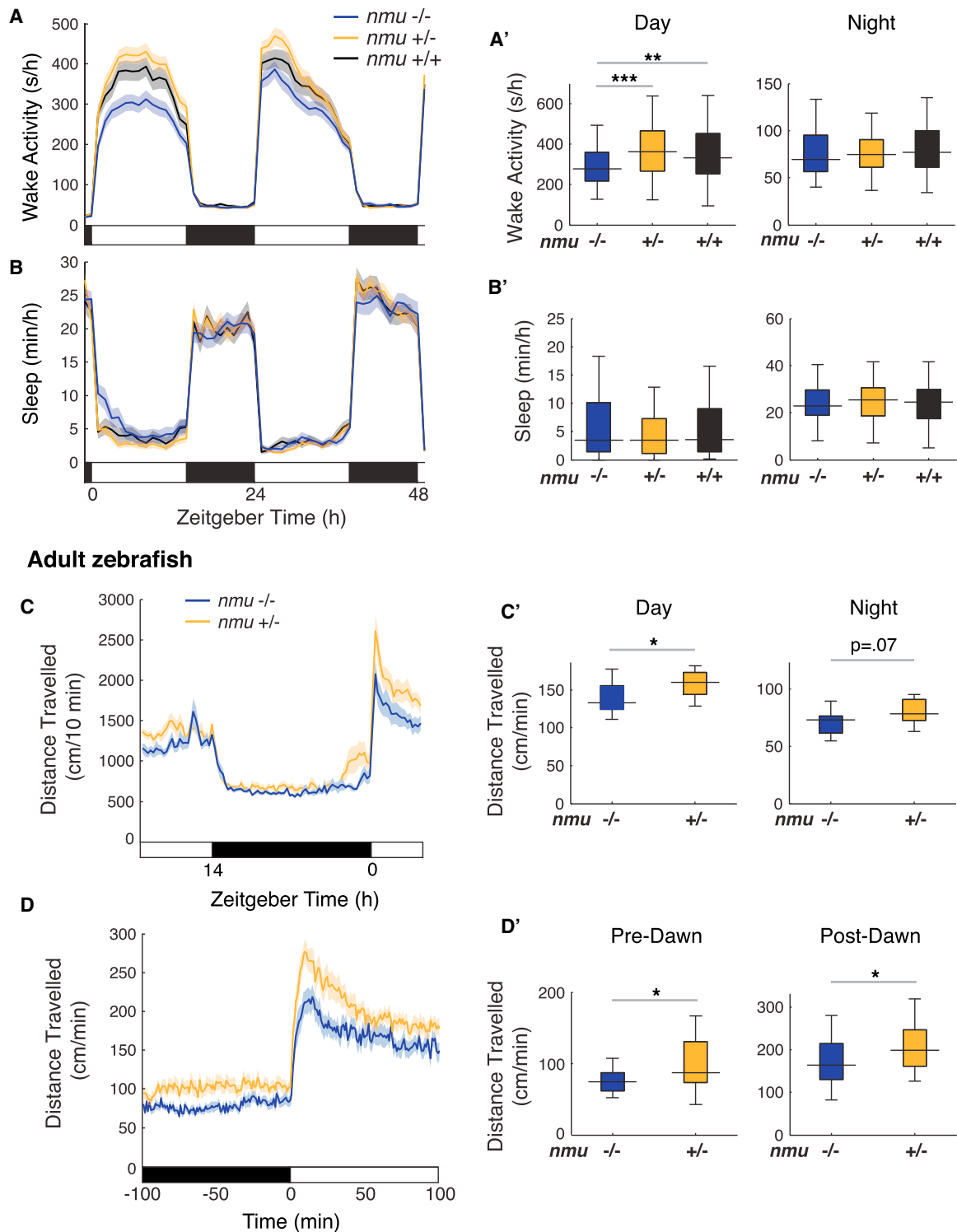Next, we set out to delineate the downstream genetic and neural circuit mechanisms linking Nmu signaling via Nmur2 to a behavioral output. Given that Nmu induces hyperactivity in rodents and zebrafish, one candidate downstream pathway is the hypothalamic-pituitary-adrenal (HPA) axis, which mediates stress behavior. HPA axis signaling is initiated by *corticotropin releasing hormone* (*crh*)-expressing neurons of the paraventricular nucleus (PVN) of the hypothalamus and eventually activates adrenal cells that affect glucocorticoid (e.g., cortisol) signaling. Indeed, it was hypothesized that Nmu-induced hyperactivity is mediated by the HPA axis, based on the observation that Nmu administration does not induce locomotor activity in *crh* mutant mice (Hanada et al., 2001). However, it was not determined which *crh*-expressing neurons were responsible for this effect. The hypothalamic-pituitary-interrenal (HPI) axis of zebrafish is homologous to the mammalian HPA axis, with cortisol/glucocorticoid receptor signaling conserved as the key effector of the HPA/HPI axis stress response (Alsop and Vijayan, 2008; De Marco et al., 2013; Wendelaar Bonga, 1997). To directly test the hypothesis that Nmu-induced hyperactivity is mediated by the HPA/HPI axis, we analyzed the effect of Nmu overexpression in zebrafish possessing a null mutation in the *glucocorticoid receptor* (*gr*) (Ziv et al., 2013), the classical target of the HPA/HPI cascade. Surprisingly, we found that the Nmu overexpression phenotype persisted in *gr*−/− larvae (Figure 6). This result suggests that Nmu-induced locomotor activity is not mediated by the HPA/HPI axis, but rather acts via an alternative Crh-dependent mechanism.

Nmu-Induced cfos Activation in Putative Brainstem Arousal Systems Expressing crh

To generate new hypotheses of Nmu downstream signaling mechanisms, we used *cfos* expression as a readout for neurons that are stimulated in response to Nmu signaling. We found that at 1 hr post-heat shock, Nmu overexpression induced *cfos* expression in several distributed cell populations, particularly in the forebrain and in discrete brainstem areas (Figure 7A). Notably, some *cfos*-positive neurons mapped closely to *nmur2*-positive neurons in the brainstem (compare Figures 7A and 2L). We observed comparatively little *cfos* expression in brains of non-transgenic siblings that underwent identical heat shock treatment (Figure 7B), suggesting that the *cfos* labeling observed in *Tg(hs:Nmu_Dr)* larvae is specific to Nmu overexpression.
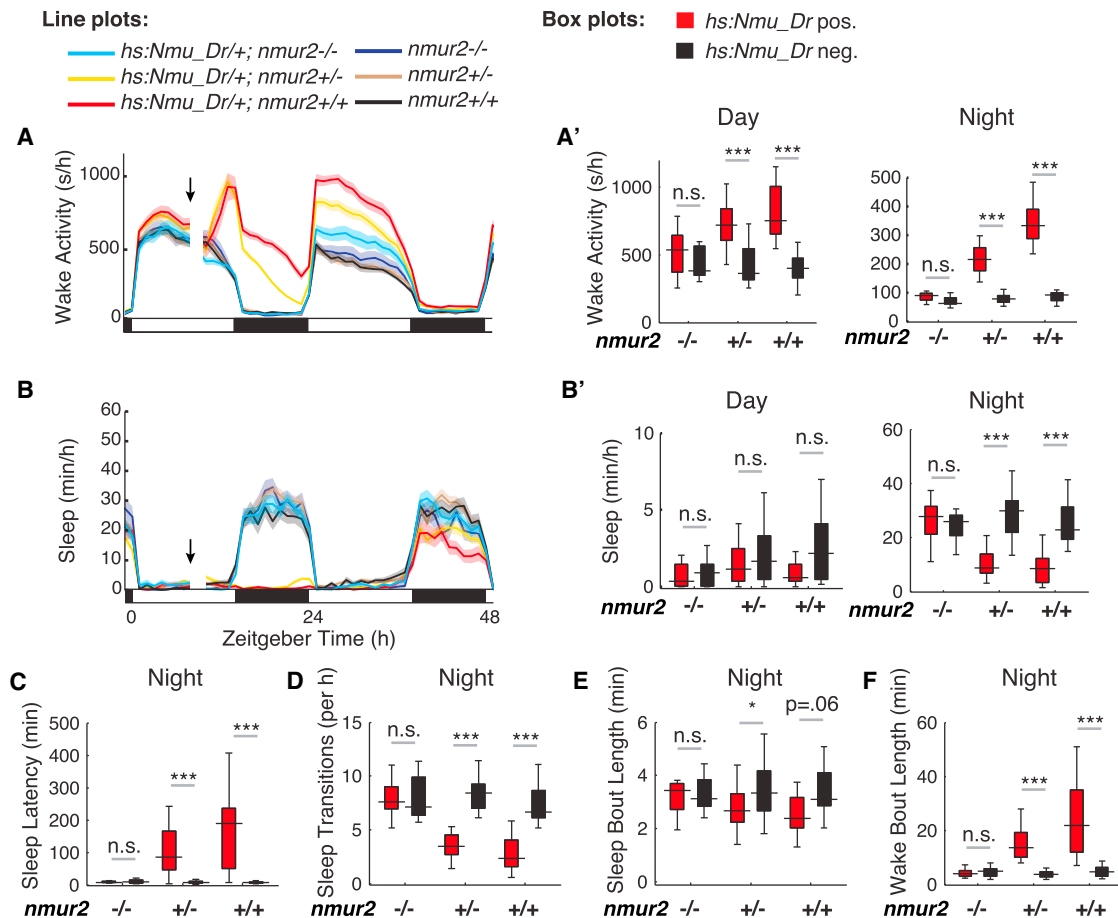
The anatomical location of one nucleus of *cfos*-positive neurons suggested that they might belong to brainstem populations that promote arousal in vertebrates. In mammals, extrahypothalamic *crh* expression has been observed in brainstem arousal nuclei, including the locus coeruleus and parabrachial nucleus (Morin et al., 1999). Thus, we hypothesized that Nmu overexpression might activate some of these brainstem *crh*-expressing neurons in zebrafish. To test this hypothesis, we performed double fluorescent ISH on brains from *Tg(hs:Nmu_Dr)* larvae and found that the brainstem *cfos*-positive neurons colocalized with *crh* (Figures 7C and 7D). Interestingly, colocalization of *cfos* and *crh* was specific to the brainstem, whereas *cfos* did not colocalize with *crh*-positive neurons in the zebrafish preoptic nucleus (Figure 7C), which is homologous to the mammalian hypothalamic PVN component of the HPA axis (Herget et al., 2014). This result is

Figure 4. *nmu* Is Required for Normal Locomotor Activity Levels in Larval and Adult Zebrafish

nmu−/− larvae exhibit reduced wake activity (A) during the day (A′) but not at night (A′) compared to their *nmu*+/− and *nmu*+/+ siblings. *nmu*−/− larvae lack a detectable sleep phenotype (B and B′). Three-month-old *nmu*−/− adults also exhibit reduced activity compared to their *nmu*+/− siblings (C) during the day (C′) and during night-to-day transitions (D and D′). Data in adult line graphs are averaged in 10 (C) or 1 (D) minute bins. Adult animals were derived from an *nmu*−/− to *nmu*+/− mating. Averaged data for all night to day transitions is shown. Line plots indicate mean ± SEM. Number of subjects: (A and B) *nmu*−/− (n = 57), *nmu*+/− (n = 82), *nmu*+/+ (n = 51). (C and D) *nmu*−/− (n = 14), *nmu*+/− (n = 15). *p < 0.05, **p < 0.01, ***p < 0.001, Kruskal-Wallis test (A′–D′) followed by the Steel-Dwass test for pairwise multiple comparisons (A′ and B′).
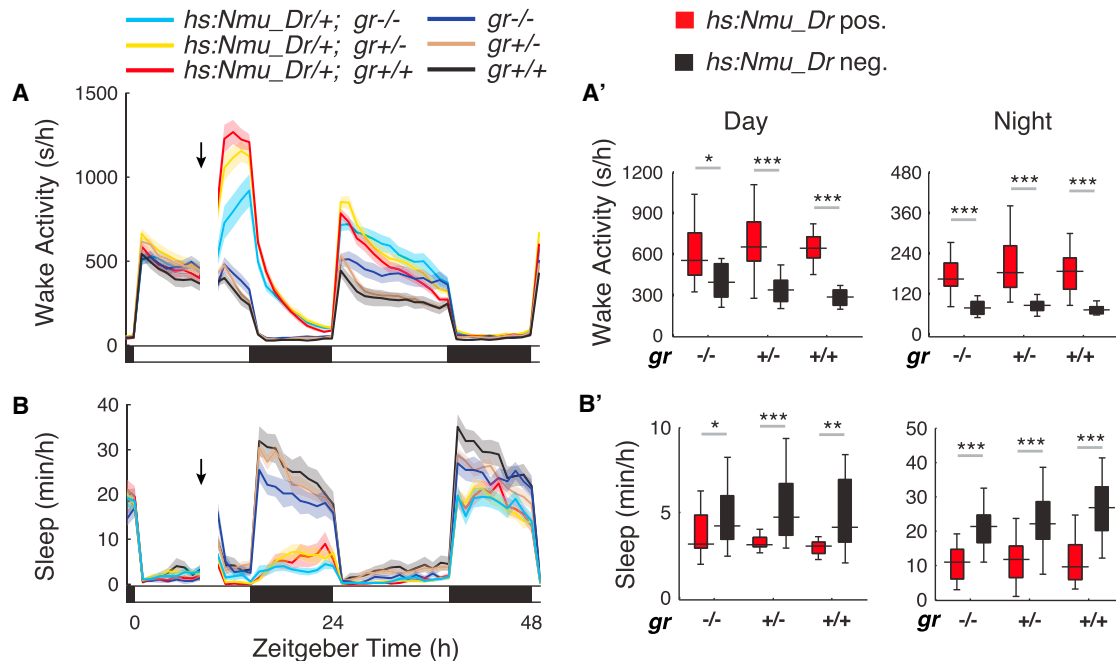
Figure 5. Nmu-Induced Hyperactivity and Insomnia Requires *nmur2*

Nmu overexpression in *nmur2*+/+ larvae (compare red and black traces in A and B) or *nmur2*+/− larvae (compare yellow and brown traces in A and B) increases wake activity (A and A′), suppresses sleep (B and B′), increases latency to first sleep at night (C), and induces fewer (D) and shorter (E) sleep bouts, and longer wake bouts (F) compared to *hs:Nmu_Dr*-negative siblings in *nmur2*+/+ and *nmur2*+/− larvae. In contrast, Nmu overexpression has no significant effect on these behavioral parameters in *nmur2*−/− larvae (compare cyan and blue traces in A, B, and compare *hs:Nmu_Dr* positive, red boxes to *hs:Nmu_Dr* negative, black boxes, for each *nmur2* genotype in box plots). Arrows indicate time of heat shock. Box plots quantify data from post-heat shock day (A′ and B′) or night periods (A′, B′, and C–F). Line plots indicate mean ± SEM. Number of subjects: *hs:Nmu/+; nmur2*−/− (n = 24), *hs:Nmu/+; nmur2*+/− (n = 42), *hs:Nmu/+; nmur2*+/+ (n = 25), *nmur2*−/− (n = 13), *nmur2*+/− (n = 39), *nmur2*+/+ (n = 23). *p < 0.05, ***p < 0.001, n.s. p > 0.05, Kruskal-Wallis test followed by the Steel-Dwass test for pairwise multiple comparisons. For *hs:Nmu/+* neg. larvae, all comparisons between *nmur2*−/−, *nmur2*+/− and *nmur2*+/+ siblings were n.s. (p > 0.05).

consistent with the persistence of the Nmu overexpression phenotype in the *gr* mutant and provides further evidence that the HPA/HPI axis is not a critical mediator of Nmu-induced arousal. Rather, our data are consistent with Nmu promoting arousal via brainstem arousal systems expressing *crh*.

To address whether there is a direct signaling pathway between the Nmu system and Crh brainstem populations, we performed double fluorescent ISH on WT larvae to assess possible colocalization of *nmur2* with the *crh* brainstem populations (Figure 7E). Expression of *nmur2* and *crh* in this region exhibits some overlap, and at least some *nmur2*-expressing cells in this region express *cfos* following Nmu overexpression (Figure 7F), but the majority of the *nmur2* expression does not colocalize with *crh* and instead resides in a closely neighboring population. These results suggest both direct and indirect pathways between the Nmu and Crh systems.

Finally, we examined the functional interaction between Nmu and Crh signaling. Based on data from the present study and previous mammalian studies (Hanada et al., 2001), we hypothesized that Crh mediates Nmu-induced arousal in larval zebrafish. We tested this hypothesis by blocking Crh signaling in the Nmu gain-of-function transgenic line. Indeed, we found that the Crh receptor 1 (Crhr1) antagonist NBI 27914, applied immediately following heat shock, blocked the Nmu overexpression-induced phenotype in a dose-dependent manner during the day following heat shock (Figures 7G and 7H). Notably, the antagonist significantly attenuated the Nmu overexpression-induced phenotype at concentrations that did not affect non-transgenic siblings (Figure 7H), suggesting that Nmu overexpressing larvae are more sensitive to the antagonist (p = 2.9 × 10^{-6} genotype × drug interaction, two-way ANOVA) and consistent with the hypothesized role of Crh signaling in Nmu-induced arousal. At 5 μM, the

Figure 6. Nmu Overexpression Phenotypes Do Not Require a Key Signaling Component of HPA Axis

(A–B′) Nmu overexpression phenotypes persist in $gr-/-$ larvae. Line plots indicate mean ± SEM. Number of subjects: $hs:Nmu/+; gr-/-$ (n = 29), $hs:Nmu/+; gr+/-$ (n = 46), $hs:Nmu/+; gr+/+$ (n = 29), $gr-/-$ (n = 21), $gr+/-$ (n = 44), $gr+/+$ (n = 17). *p < 0.05, **p < 0.01, ***p < 0.001, Kruskal-Wallis test followed by the Steel-Dwass test for pairwise multiple comparisons. For $hs:Nmu/+$ neg. larvae, all comparisons between $gr-/-$, $gr+/-$, and $gr+/+$ siblings were n.s. (p > 0.05).

Crhr1 antagonist also reduced locomotor activity in non-transgenic sibling larvae (Figures 7G and 7H), consistent with a requirement for Crh signaling to maintain normal arousal levels. Taken together, our anatomical and functional data suggest that Nmu-induced arousal is mediated by Crh signaling in the brainstem.

DISCUSSION

An Overexpression Screen to Identify Genes that Regulate Sleep

By combining injection-based gene overexpression with high-throughput locomotor activity assays, we developed a novel and cost-effective approach to identify molecular regulators of zebrafish sleep/wake behaviors. Using a heat-shock inducible promoter to broadly overexpress secreted proteins overcomes several limitations of traditional screens. For example, broad overexpression can produce more robust and reproducible phenotypes than tissue-restrictive approaches and haplo-insufficient mutagenesis, as well as chemical mutagenesis approaches that induce thousands of mutations in each animal. Inducible gene overexpression also increases the sensitivity of the screen because phenotypes are compared before and after gene overexpression in the same animals. Furthermore, our inducible approach circumvents developmental compensatory mechanisms, which may mask phenotypes, and allows for the analysis of genes with developmental functions.

A significant limitation of the screen was a high false positive rate, with only 10 of the 60 hits from the primary screen produc-ing the expected phenotype in stable transgenic lines. This high false positive rate is partly due to our relatively relaxed cutoff in the primary screen. However, had we used a more stringent threshold, we would have missed key genes such as Nmu and potentially biased the results for strong, non-physiological effectors. It is unclear if false positives were also due to poor reproducibility of the injection procedure, the number of larvae tested for each gene, the variable nature of sleep/wake behavioral phenotypes, or the use of human genes.

The screen identified Nmu as a conserved regulator of locomotor activity in vertebrates. We further identified a role for Nmu and its receptor Nmur2 in regulating sleep/wake architecture in zebrafish. In contrast to a previous hypothesis that Nmu-induced locomotor activity is mediated by the HPA/HPI stress axis, our results suggest that brainstem Crh neurons mediate the function of Nmu in vertebrate sleep/wake regulation (Figure 8). The interaction between the Nmu system and brainstem Crh arousal systems represents a novel vertebrate arousal circuit.

Conserved Molecular Circuitry and Motor Function of Nmu

We found that the Nmu peptide sequence and expression pattern in the brain are well-conserved from mammals to zebrafish. Furthermore, we found that Nmu overexpression promotes wakefulness and hyperarousal in zebrafish, consistent with the increased physical activity observed in mammals following acute administration of Nmu peptide (Nakazato et al., 2000; Novak et al., 2006, 2007; Peier et al., 2009; Semjonous et al., 2009;

Wren et al., 2002). We also observed arousal defects in *nmu* mutant zebrafish larvae and adults. Larval zebrafish *nmu* mutants are less active during the day, suggesting that *nmu* is required for normal daytime arousal levels. The adult zebrafish *nmu* mutant phenotype is most prominent just prior to and following the onset of light in the morning, suggesting that Nmu is particularly important in promoting the transition from nighttime sleep to daytime wakefulness. In rodents, the role of endogenous *nmu* in sleep and arousal is unclear, as one study observed locomotor defects in *nmu* mutant mice (Hanada et al., 2004), whereas a recent study using the same strain did not (Lee et al., 2015).

The two mammalian Nmu receptors are also conserved in zebrafish, which have two orthologs of mammalian *nmur1* (*nmur1a* and *nmur1b*), and one ortholog of mammalian *nmur2*. Similar to mammals (Gartlon et al., 2004), zebrafish *nmur2* is enriched in widespread yet specific regions of the brain, whereas *nmur1a* is sparsely expressed in the brain and *nmur1b* expression is undetected up to 120 hpf. The effects of Nmu peptide administration on physical activity are absent in *nmur2* knockout mice, indicating that Nmur2 is required to mediate Nmu-induced locomotor activity in rodents (Peier et al., 2009; Zeng et al., 2006). Similarly, we find that locomotor phenotypes induced by Nmu overexpression are abolished in *nmur2−/−* zebrafish but persist in *nmur1a−/−* and *nmur1b−/−* zebrafish. Thus, the role of Nmur2 as the primary mediator of Nmu-induced locomotor activity is conserved from mammals to zebrafish. We furthermore extend the behavioral roles for Nmur2 to regulating arousal and insomnia phenotypes induced by Nmu overexpression.

Intriguingly, *nmur2* knockout mice were reported to not exhibit locomotor activity phenotypes, although behavior was only monitored for short time intervals during the day (Peier et al., 2009; Zeng et al., 2006). Although we observed little or no locomotor activity phenotypes in *nmur2* mutant zebrafish larvae, we did observe reduced locomotor activity in adult zebrafish, with the strongest effects observed in the morning after lights on, consistent with the zebrafish *nmu* mutant adult phenotype. Therefore, the reported differences in *nmur2−/−* locomotor phenotypes between rodents and zebrafish may be due to the time of day when observations are made.

In addition to effects on behavior, we observed that both the *nmu* and *nmur1a* mutants, but not the *nmur1b* or *nmur2* mutants, are significantly smaller as adults. These observations suggest that Nmur1a and Nmur2 mediate distinct aspects of Nmu function. The small size of adult zebrafish *nmu* and *nmur1a* mutants is surprising given that the mouse *nmu* mutant is obese (Hanada et al., 2004). However, the mouse *nmur1* mutant does not exhibit growth abnormalities and the mouse *nmur2* mutant exhibits a growth defect that correlates with decreased feeding (Peier et al., 2009). It may be that differences in feeding schedules contribute to differences in Nmu mutant phenotypes; zebrafish are given a restricted feeding regimen, whereas mice were fed ad libitum.

Role of Nmu in Modulating Spontaneous versus Stimulus-Evoked Arousal
Our analyses of sleep/wake architecture refine the previously established role of Nmu in regulating locomotor activity in verte-
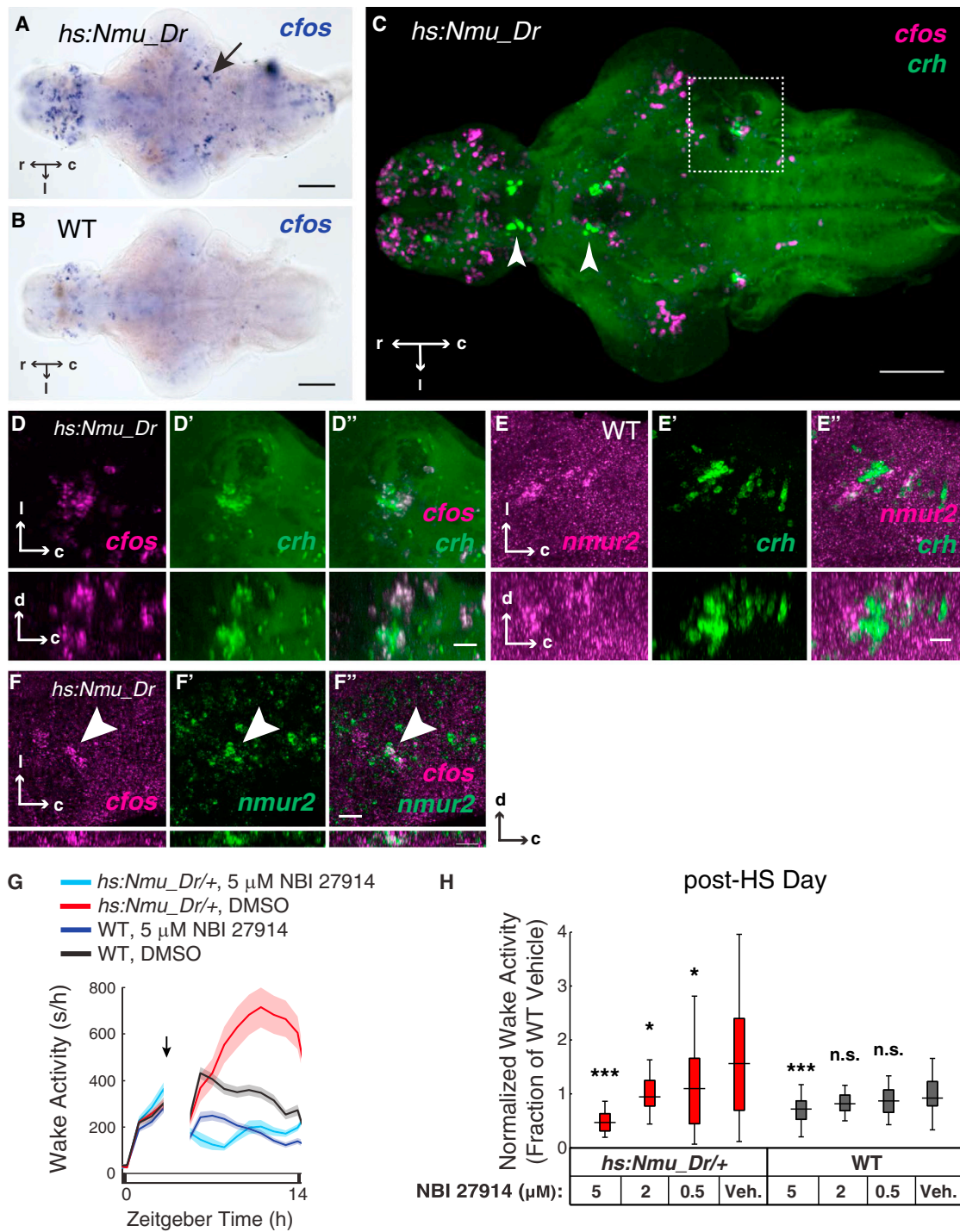
brates. Nmu overexpression consolidates waking bouts into periods of prolonged hyperactivity and shortens and reduces initiations to sleep, similar to insomnia. The effect of Nmu on sleep/wake architecture has not been extensively examined in other species, but our results are consistent with the short-term effect of acute intracerebroventricular injection of Nmu in rats, namely prolonged wakefulness and disrupted sleep (Ahnaou and Drinkenburg, 2011).

We also examined effects of Nmu overexpression on stimulus-evoked arousal using a simple behavioral paradigm to evoke both an immediate and delayed arousal response. These results suggest that while Nmu can modulate both stimulus-evoked and spontaneous forms of arousal, it does so in a manner that heightens and enhances the maintenance of ongoing arousal behavior and conversely constricts immediate responses to environmental stimuli. Future studies are required to dissect the downstream mechanisms by which Nmu differentially modulates these immediate and delayed arousal responses.

A Revised Model for Nmu Signaling Mechanisms
Multiple lines of evidence point to a role for Crh as an essential effector of hyperactivity and anorexia of Nmu-treated rodents (Hanada et al., 2001, 2003). Similarly in zebrafish, we found that Nmu-induced hyperactivity is blocked by Crh receptor antagonism. Based on the observation that Crh signaling mediates Nmu-induced activity in rodents, it was proposed that Nmu's effects are mediated by the HPA axis (Hanada et al., 2001). However, the requirement for the glucocorticoid receptor (GR), the ultimate effector of Crh signaling in the HPA axis, for Nmu-mediated arousal has not been tested. In contradiction to this hypothesis, we found that the Nmu overexpression phenotype in zebrafish does not require a functional GR. Furthermore, Nmu overexpression does not activate *cfos* expression in Crh neurons in the preoptic area, the zebrafish homolog of the mammalian PVN (Herget et al., 2014). Our results suggest that Nmu overexpression does not activate the HPA axis to promote arousal, which concords with previous findings in rodents that chronic administration of Nmu does not elevate plasma corticosterone levels (Peier et al., 2009), although acute administration of Nmu has been reported to increase corticosterone levels (Wren et al., 2002). Furthermore, *nmur2* mouse mutants do not exhibit abnormal basal or stress-induced corticosterone levels (Peier et al., 2009).

Our data on Nmu-induced *cfos* expression support a revised model that Nmu-induced sleep/wake phenotypes are mediated by HPA/HPI axis-independent, extrahypothalamic Crh neurons (Figure 8). We found that a Crhr1 antagonist reduced baseline locomotor activity in WT larvae, suggesting that Crh signaling is required for normal arousal levels and not just behavioral responses to stress. In mammals, Crh signaling regulates both spontaneous and stimulus-evoked sleep/wake behaviors (Opp, 1995). Also, alterations in Crh-mediated sleep/wake behavior of rodents can occur without affecting corticosterone signaling (Chang and Opp, 2002), and Crh administration increases locomotor activity in hypophysectomized rats (Eaves et al., 1985), indicating that Crh signaling can regulate arousal independently of the HPA axis. Interestingly, the effects of Nmu administration on promoting spontaneous arousal behaviors while reducing

Figure 7. Nmu Overexpression Activates Brainstem *crh* Neurons and the Nmu Overexpression Locomotor Phenotype Requires Crh Receptor 1 Signaling
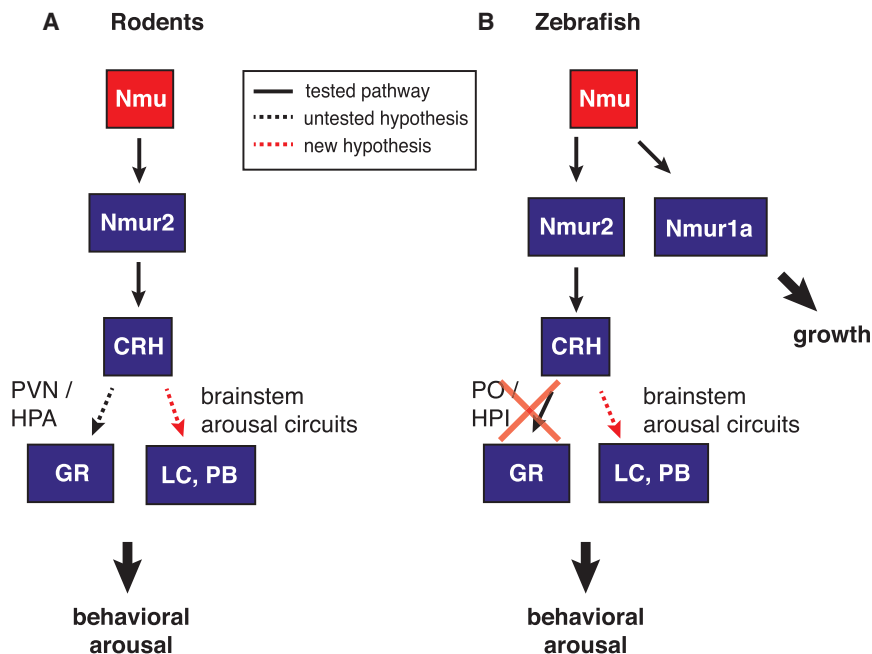
(A and B) ISH analysis of *cfos* expression 1 hr after heat-shock induction of Nmu overexpression in a 5 dpf *hs:Nmu_Dr/+* transgenic zebrafish brain (A) and in an identically treated non-transgenic sibling (B). Arrow indicates *cfos*-expressing brainstem populations.

(C–D'') Double fluorescent ISH reveals brainstem colocalization of *cfos* and *crh* expression following Nmu overexpression (dotted box is magnified in D), whereas diencephalic *crh* expression does not colocalize with *cfos* (arrowheads). The brainstem populations in (C) are presented in separate and overlaid confocal channels at higher magnification dorsal (D–D'', top) and orthogonal (D–D'', bottom) views to demonstrate colocalization.

(E–E'') Double fluorescent ISH reveals sparse colocalization of *nmur2* and *crh* expression in the same brainstem populations and in the same views as shown in (D)–(D'').

(legend continued on next page)

A   Rodents

B   Zebrafish

Figure 8. Newly Tested and Revised Hypotheses of Nmu Signaling in Sleep/Wake Behavior

(A) Rodent studies propose that Nmu-induced behavioral phenotypes are mediated by Crh neurons in the paraventricular nucleus of the hypothalamus (PVN). The PVN is the starting point of the hypothalamic-pituitary-adrenal (HPA) axis, in which binding of glucocorticoids to the glucocorticoid receptor (GR) is the final step of the HPA signaling cascade.

(B) Our experiments in zebrafish show a conserved functional Nmu/Nmur2/Crh signaling pathway but contradict a role for HPA/HPI axis in zebrafish Nmu signaling by demonstrating that Nmu's actions do not require the GR. We put forth the revised hypothesis that Nmu-induced arousal phenotypes are mediated by extra-hypothalamic sources of Crh that participate in brainstem arousal systems, such as the locus coeruleus (LC) and parabrachial nucleus (PB). We also show that Nmu and Nmur1a are required for the normal growth of adult zebrafish.

acute, "stimulus-on" responses in our light flash-evoked assay of zebrafish arousal parallels the roles of Crh in rodents to enhance spontaneous activity but to decrease acute locomotor activity responses to novel situations (Koob and Heinrichs, 1999). We found that Nmu overexpression results in activation of brainstem Crh nuclei, as well as nuclei in the forebrain. Indeed, Crh is expressed in the locus coeruleus and parabrachial nuclei of mammals (Morin et al., 1999) and has been shown to directly activate noradrenergic neurons of the locus coeruleus (Jedema and Grace, 2004) which in turn send ascending arousal signals to the forebrain (Berridge and Waterhouse, 2003). We speculate that Nmu-induced forebrain activation may result from activation of Crh neurons participating in the ascending pathways of brainstem arousal nuclei.

In summary, our results identify Nmu as a regulator of sleep/wake behavior in the zebrafish. Using a combination of behavioral, genetic, pharmacological, and anatomical approaches, we further dissected this neuropeptidergic signaling pathway and propose a novel mechanism for the regulation of vertebrate behavioral arousal. More generally, our work establishes an effective and efficient genetic screening strategy and provides a community resource for future studies to screen for effectors of vertebrate behavior and development.

EXPERIMENTAL PROCEDURES

Detailed methods are in Supplemental Experimental Procedures.

Behavioral Experiments

Larval zebrafish behavioral experiments were performed and analyzed as described (Prober et al., 2006). In brief, 4–5 dpf larvae were transferred to 96-well plates and housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and illuminated with white lights from 9 a.m. to 11 p.m. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5°C. The movement of each larva was measured at 15 Hz and the duration of movement was recorded with an integration time of 1 min for most experiments, except in the arousal assay the integration time was 1 s.

Statistical Analyses

Statistical tests were performed using MATLAB and JMP. In all statistical tests, the significance threshold was set to $p < 0.05$. Asterisks in figures denote statistics for pairwise comparisons using the Steel-Dwass method for multiple comparisons following significant Kruskal-Wallis tests. Box plots indicate the median value (solid black line), 25th and 75th percentiles (box), and data range (whiskers). "Day" box plots and statistics combine all daytime periods, and "Night" box plots and statistics combine all nighttime periods displayed in the corresponding line graph. For heat-shock overexpression experiments, box plots and statistics were generated from post-heat shock time points.

(F) Following Nmu overexpression, some *nmur2*-expressing cells colocalize with *cfos* in the same brainstem populations and in the same views as shown in (E–E''). Scale bars = 100 μm in (A)–(C) and 20 μm in (D)–(F). Images are maximum intensity projections of optical stack thickness = 300 μm (C), 82 μm (D), 85 μm (E), and 16 μm (F).

(G) 5 μM NBI 27914, a Crhr1 antagonist, or vehicle (0.05% DMSO) was applied to *hs:Nmu_Dr*/+ larvae and their WT siblings immediately after a 1 hr heat shock at 12 p.m. Line plot indicates mean ± SEM. Number of subjects: *hs:Nmu_Dr*/+, 5 μM NBI 27914 (n = 40), WT, 5 μM NBI 27914 (n = 55), *hs:Nmu_Dr*/+, DMSO (n = 47), WT, DMSO (n = 49).

(H) Additional experiments performed at 2 μM and 0.5 μM NBI 27914 reveal dose-dependent inhibition of the Nmu overexpression phenotype. Data were normalized by dividing the average post-HS day waking activity of each fish by the mean value of within-experiment WT vehicle samples. Normalized values from individual samples across separate experiments were then pooled for analysis and box plots. Number of subjects: *hs:Nmu_Dr*/+, NBI 27914: 5 μM (n = 40), 2 μM (n = 25), 0.5 μM (n = 35), DMSO (n = 101); WT, NBI 27914: 5 μM (n = 55), 2 μM (n = 23), 0.5 μM (n = 32), DMSO (n = 103). *$p < 0.05$, **$p < 0.01$, n.s. $p > 0.05$, Kruskal-Wallis test followed by the Steel-Dwass test for pairwise multiple comparisons of each drug condition against its same-genotype vehicle control.

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