A Perfect Day for Zebrafish

Neuromodulation of Sleep in a Diurnal Vertebrate

Thesis by

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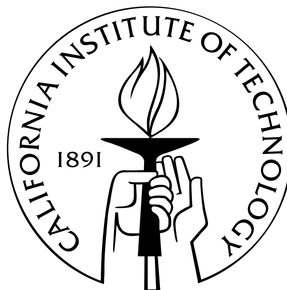

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for my parents

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Abstract

EVERY day, we shift among various states of sleep and arousal to meet the many demands of our bodies and environment. A central puzzle in neurobiology is how the brain controls these behavioral states, which are essential to an animal's well-being and survival. Mammalian models have predominated sleep and arousal research, although in the past decade, invertebrate models have made significant contributions to our understanding of the genetic underpinnings of behavioral states. More recently, the zebrafish (*Danio rerio*), a diurnal vertebrate, has emerged as a promising model system for sleep and arousal research.

In this thesis, I describe two studies on sleep/arousal pathways that I conducted using zebrafish, and I discuss how the findings can be combined in future projects to advance our understanding of vertebrate sleep/arousal pathways. In the first study, I discovered a neuropeptide that regulates zebrafish sleep and arousal as a result of a large-scale effort to identify molecules that regulate behavioral states. Taking advantage of facile zebrafish genetics, I constructed mutants for the three known receptors of this peptide and identified the one receptor that exclusively mediates the observed behavioral effects. I further show that the peptide exerts its behavioral effects independently of signaling at a key module of a neuroendocrine signaling pathway. This

finding contradicts the hypothesis put forth in mammalian systems that the peptide acts through the classical neuroendocrine pathway; our data further generate new testable hypotheses for determining the central nervous system or alternative neuroendocrine pathways involved.

Second, I will present the development of a chemigenetic method to non-invasively manipulate neurons in the behaving zebrafish. I validated this technique by expressing and inducing the chemigenetic tool in a restricted population of sleep-regulating neurons in the zebrafish. As predicted by established models of this vertebrate sleep regulator, chemigenetic activation of these neurons induced hyperactivity, whereas chemigenetic ablation of these neurons induced increased sleep behavior. Given that light is a potent modulator of behavior in zebrafish, our proof-of-principle data provide a springboard for future studies of sleep/arousal and other light-dependent behaviors to interrogate genetically-defined populations of neurons independently of optogenetic tools.

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Chapter 1

Introduction

The following article is reproduced, with slight adaptation, for this thesis: Chiu CN and Prober DA (2013) Regulation of zebrafish sleep and arousal states: current and prospective approaches. Front. Neural Circuits 7:58. doi:10.3389/fncir.2013.00058

Animals engage in diverse activities that require adaptive changes in behavior. A fundamental goal in neuroscience is to understand how the brain enables animals to make dynamic changes in behavioral state in response to changing internal or environmental demands. A particularly striking example of such a change in behavioral state is the switch between sleep and wakefulness. Once awake, animals must further modulate arousal levels, for example transitioning between inattentive and attentive states, as required for the task at hand. Underscoring the significance of these behavioral states, sleep and arousal states are conserved across the animal kingdom, from worms and flies to fish and humans (Allada & Siegel, 2008, Cirelli & Tononi, 2008). Despite the prevalent and severe consequences of sleep and arousal disorders (Mahowald & Schenck, 2005), the mechanisms that regulate behavioral states and transitions between states remain mysterious.

Theories that account for the regulation of sleep and arousal states span the hier-

archy of biological organization, from organismal physiology, behavior, and cognition to neurons and neural ensembles, and more recently to genetic and molecular mechanisms (Hobson & Pace-Schott, 2002, Pace-Schott & Hobson, 2002). The zebrafish, which offers experimental advantages at many levels, is well suited to contribute to our understanding of these states. These advantages include a simplified yet conserved vertebrate brain, facile genetics, an increasingly well-characterized behavioral repertoire, amenability to pharmacological and high-throughput assays, and optical transparency for *in vivo* visualization of the brain (Lieschke & Currie, 2007). The zebrafish is also gaining traction as a useful system for circuit neuroscience (Friedrich et al., 2010, McLean & Fetcho, 2011, Portugues et al., 2013).

To introduce my thesis work, I will survey key concepts and open questions in the field of sleep and arousal regulation, and then examine current approaches to identifying these behavioral states in zebrafish. To exemplify these concepts and the issues that arise when using zebrafish to study neuromodulation of sleep and arousal, I will focus my discussion on studies that explore the role of hypocretin, an important mammalian neuromodulator of sleep and arousal, in regulating zebrafish behavioral state.

1.1 Regulation of sleep and arousal: key concepts and problems

In the early 20th century, the neurologist Constantin von Economo examined encephalitis patients who suffered from profound sleep disorders, and he discovered that excessive sleepiness was associated with a specific pattern of brain lesions located at the junction of the brainstem and forebrain, whereas insomnia was associated with lesions in a nearby, more anterior region (Von Economo, 1930). Subsequently, Moruzzi, Magoun and others found that sleep or arousal could be induced by lesion or electrical activation along a subcortical pathway ascending from the brainstem (Moruzzi & Magoun, 1949). These findings advanced the idea that sleep and arousal states are actively generated and maintained by the brain. The main subcortical regions identified by von Economo and others (brainstem, posterior hypothalamus, basal forebrain) are now known to contain distinct aminergic and peptidergic cell populations (Saper et al., 2005). These systems promote arousal via ascending projections that increase forebrain excitation as well as descending brainstem and spinal cord projections that increase muscle tone and sensorimotor function (Jones, 2003).

Neuromodulatory systems that promote arousal include (refer to Figure 1.1):

- noradrenergic neurons of the locus coeuleus, located in the pontine brainstem
- serotonergic neurons of the raphe nuclei, located in the midbrain
- dopaminergic neurons, particularly those of the ventral periaqueductal gray

(vPAG) and ventral tegmental area of the midbrain (VTA), and also the A11 dopaminergic cell cluster, located in the hypothalamus

- histaminergic neurons of the tuberomammillary nucleus, located in the posterior hypothalamus
- hypocretin (Hcrt) neurons, located in the lateral hypothalamus
- cholinergic neurons, located in the basal forebrain and also in the pedunculopontine and laterodorsal tegmental nuclei, located in the pontine brainstem

In contrast to the many known arousal-promoting systems, attempts to identify distinct sleep-promoting cell populations have been less fruitful. One exception is the ventrolateral preoptic area (VLPO), which was identified as a cluster of cells in the basal forebrain that provides inputs to systems in the hypothalamus and brainstem that promote arousal (Saper et al., 2005). These neurons are likely the site of lesion in von Economo's insomnia patients, and subsequent work in animal models showed that VLPO lesions reduce sleep by more than half (Lu et al., 2000). VLPO neurons contain the inhibitory transmitters GABA and galanin, and these neurons are likely to promote sleep by inhibiting arousal systems. The VLPO, in turn, is directly and indirectly inhibited by arousal systems.

A crucial question is how these neural populations operate together to regulate distinct states and the transitions between them. The bistable flip-flop switch, adopted from electronics theory, is one appealing circuit-level model that can explain the rapid transition between distinct behavioral states such as sleep and waking (Saper et al.,

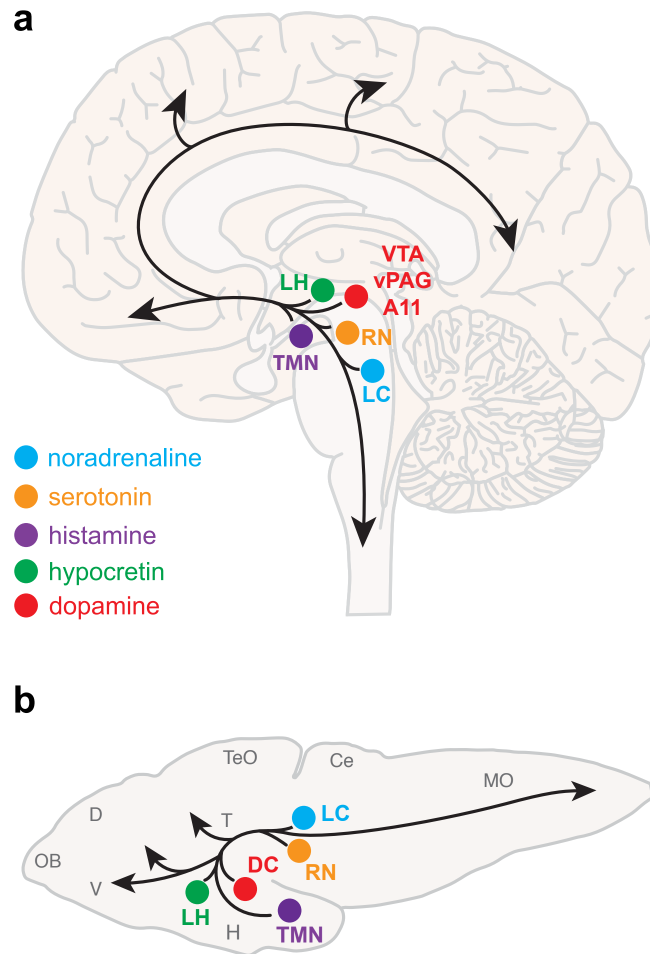

Figure 1.1: Neuromodulatory systems that promote arousal in vertebrates. The approximate locations of key neuromodulatory regions are shown for human (A) and larval zebrafish (B) brains. Arrows indicate ascending projections that increase forebrain excitation and descending projections that increase muscle tone and sensorimotor function. Abbreviations of neuromodulatory regions: LC, locus coeruleus; RN, raphe nuclei; VTA, ventral tegmental area; vPAG, ventral periaqueductal gray; A11, mammalian dopamine cell group A11; DC, dopaminergic diencephalic cluster; TMN, tuberomammillary nucleus; LH, lateral hypothalamus. Abbreviations of larval zebrafish brain anatomy: OB, olfactory bulb; D, dorsal telencephalon; V, ventral telencephalon; TeO, optic tectum; H, hypothalamus; T, thalamus; Ce, cerebellum; MO, medulla oblongata. Note human and larval zebrafish brains are not depicted to scale. Figure as originally published in Chiu & Prober (2013).

2001). The flip-flop circuit derives its features from two reciprocally inhibitory components; this could be implemented in the brain by mutual inhibition between the VLPO and arousal systems. Because the flip-flop switch is inherently unstable, the circuit model might be supplemented by additional elements (i.e. neuromodulatory systems) that serve to stabilize and sustain a wake or sleep state. For example, in mammals, the neuromodulator Hcrt might serve to stabilize sleep-wake states by promoting arousal. Indeed, loss of Hcrt signaling is a hallmark of narcolepsy, a disorder characterized by fragmented sleep-wake states.

A more phenomenological but influential model proposes that sleep is regulated by two main drives: homeostatic drive (also known as "Process S") that is regulated by internal cues and circadian drive ("Process C") that is regulated by environmental cues (Borbély et al., 1989). Genetic approaches have made remarkable contributions towards a molecular-level understanding of Process C. The core mechanism of the circadian clock is conserved across species, and consists of a network of positive and negative molecular feedback loops that can cell-autonomously maintain a 24-hour periodic rhythm (Mohawk et al., 2012, Zhang et al., 2009). In mammals, neurons of the hypothalamic suprachiasmatic nucleus (SCN) function as a "master clock" that orchestrates organismal circadian physiology and behavior, but it remains unclear whether such master clocks are a mammalian innovation or are present throughout the animal kingdom. Despite our mechanistic understanding of the circadian clock, it remains unclear how the circadian system regulates behaviors associated with sleep and wakefulness, although secreted peptides such as prokineticin 2 and transforming

growth factor alpha/epidermal growth factor appear to play key roles (Cheng et al., 2002, Foltenyi et al., 2007, Gilbert & Davis, 2009, Kramer et al., 2001, Van Buskirk & Sternberg, 2007). Our understanding of mechanisms that underlie Process S are more limited. One hypothesis is that adenosine, which accumulates as ATP energy stores are depleted during wakefulness, might serve as a signal for sleep need (Porkka-Heiskanen & Kalinchuk, 2011). Indeed, extracellular adenosine levels rise in specific regions of the mammalian brain during prolonged wakefulness and decline during sleep (Porkka-Heiskanen et al., 1997), and pharmacological activation of adenosine signaling promotes sleep (Benington et al., 1995, Hendricks & Jesuthasan, 2007, Rihel & Schier, 2012, Thakkar et al., 2001) and activates the VLPO (Gallopin et al., 2005, Scammell et al., 2001). However, the role of adenosine in sleep remains controversial, because adenosine receptor mutants exhibit relatively normal sleep/wake behaviors (Bjorness et al., 2009, Huang et al., 2005, Stenberg, 2007, Urade et al., 2003, Wu et al., 2009).

A common theme that has emerged from studies of sleep and arousal regulatory mechanisms is that they play multiple roles in animal behavior and physiology. For example, many of the key players in sleep and circadian function are linked to metabolic regulation (Adamantidis & de Lecea, 2009, Bass, 2012). Indeed, at the same time that Hcrt's link to narcolepsy was discovered (Chemelli et al., 1999, Lin et al., 1999), this peptide was also given the name orexin, because intracerebroventricular injection of the peptide induced voracious feeding in rodents (Sakurai et al., 1998). Also, there are well-documented links between obesity and abnormal circadian behaviors, includ-

ing voluntary behaviors such as shift-work (Antunes et al., 2010). This has led to the hypothesis that the circadian clock coordinates various physiological and behavioral functions in addition to sleep, such as liver function and feeding. Additionally, a number of sleep regulators have known interactions and/or overlap with regulators of immune function (Krueger, 2008) and learning and memory (Harris & Aston-Jones, 2006). Similarly, memory, attention, anxiety, and depression are among the many behavioral processes linked to arousal regulation and dysregulation (Johnson et al., 2012), and several neurological disorders, including autism and schizophrenia, are associated with sleep and arousal defects (Glickman, 2010, Pritchett et al., 2012).

Understanding how sleep and arousal are regulated might lead to new treatments for neurological diseases, as well as explain normal individual variations in sleep and arousal. Going forward, a few of the many outstanding questions regarding how behavioral states are regulated include:

- What are the undiscovered genetic and neural substrates of sleep and arousal states?
- Do conserved or diverse neural and genetic mechanisms regulate sleep and arousal throughout the animal kingdom?
- Who are the downstream effectors of Process S and Process C? For example, what are the neural and genetic pathways that link circadian input signals to a circadian behavioral output?
- How are circadian, homeostatic, and other behaviorally-relevant drives integrated at the circuit level?

- Are dynamic changes in neuromodulatory influences responsible for the transitions between different behavioral states?
- Which are the redundant and unique properties among neuromodulator systems that promote arousal? How do these systems participate in sleep and arousal and also distinct aspects of behavior? Are there anatomical and functional subdivisions within each arousal-promoting neuromodulator system?
- Can we develop or discover effective remedies for sleep and arousal-related disorders? In particular, can we learn enough about mechanism to treat specific pathologies without grossly affecting other brain functions?

Despite their tremendous contributions to sleep and arousal research, prevalent animal model systems have limitations in addressing some of these questions. For example, drawbacks of rodent model systems include the relative complexity of their nervous systems, the difficulty of monitoring the activity of genetically identified neurons during behavior, and a nocturnal sleep/wake pattern that differs from diurnal humans. Also, the long generation time, small litter size, and expense make the rodent an unwieldy model for large-scale behavioral screening. On the other hand, the fruit fly and worm are particularly amenable to genetic screens, but their nervous systems lack anatomical structures and some neuromodulators analogous to mammals. In this light, the zebrafish, a diurnal vertebrate with cutting-edge genetic and *in vivo* neuroimaging capabilities and a successful track-record in high-throughput behavioral screens, is an excellent system to complement the advances made using mammalian

and invertebrate model systems.

1.2 Analysis of zebrafish behavioral states

After only a few days of development, larval zebrafish begin to swim around in their environment, typically in brief, phasic locomotor episodes. High-speed infrared video capture combined with computational image analyses have been used to quantitatively describe specific locomotor behaviors in larval zebrafish. For example, by measuring values for indicator variables, such as those characterizing an animal's posture (tail bend location and amplitude, turning angle, yaw) and timing (tail-beat frequency, swimming speed), it is possible to objectively define and differentiate basic locomotor modules such as scoots, burst swims, routine-turns, and escape-turns (Budick & O'Malley, 2000). Similarly, an animal's behavioral state can be defined as a *recurring, temporally enduring constellation of values of a set of indicator variables of the organism* (Steriade & McCarley, 2005). Sleep and waking states are typically defined in this manner. In humans and other mammals, these states can be distinguished by obvious differences in behavior, but they are more conveniently identified by objective electrophysiological measures that correlate with behavioral state, such as the electroencephalogram (EEG), which measures cerebral electrical activity (Berger, 1929). In fact, the EEG and similar measures of global brain activity reveal physiological subdivisions within sleep and waking, suggesting that they are not unitary states (Lin & Gervasoni, 2008). The use of physiological criteria is a practical and standardizable approach to defining behavioral states (Datta & Hobson, 2000,

Rechtschaffen & Kales, 1968), although it is associated with the hazards of inferring cause from inappropriate or indirect measures (Hobson & Steriade, 1986). With this in mind, behavioral measures are most appropriate for describing behavioral states, whereas physiological measures of sleep and arousal, while experimentally convenient in some animals, should be carefully regarded as correlative.

There are also important methodological issues to address when measuring behavioral states in non-human animals such as zebrafish. First, tracking of individual rather than groups of animals is ideal for resolving the temporal structure of sleep and arousal states. Second, the impact of genetic variations (Valatx et al., 1972) and prior experiences (Ganguly-Fitzgerald et al., 2006) on sleep and arousal must be carefully controlled for reproducible measurements. Fortunately, with care, these issues can be reasonably addressed using zebrafish (Figure 1.2). Large clutches of embryos allow for experimental comparisons of siblings that are raised and tested together in identical conditions. Because they can survive on their yolk sac for the first week of development, the confounding effects of variable feeding behavior are avoided. Most importantly, the small size of larval zebrafish (∼4mm in length) allows for simultaneous behavioral tracking of individually-housed animals in a 96-well plate.

1.2.1 Zebrafish sleep states

In non-mammalian and non-avian animal model systems, sleep is defined according to several behavioral criteria (Campbell & Tobler, 1984): 1) quiescent state regulated by a circadian rhythm, 2) reduced sensory responsiveness, and 3) homeostatic regulation.

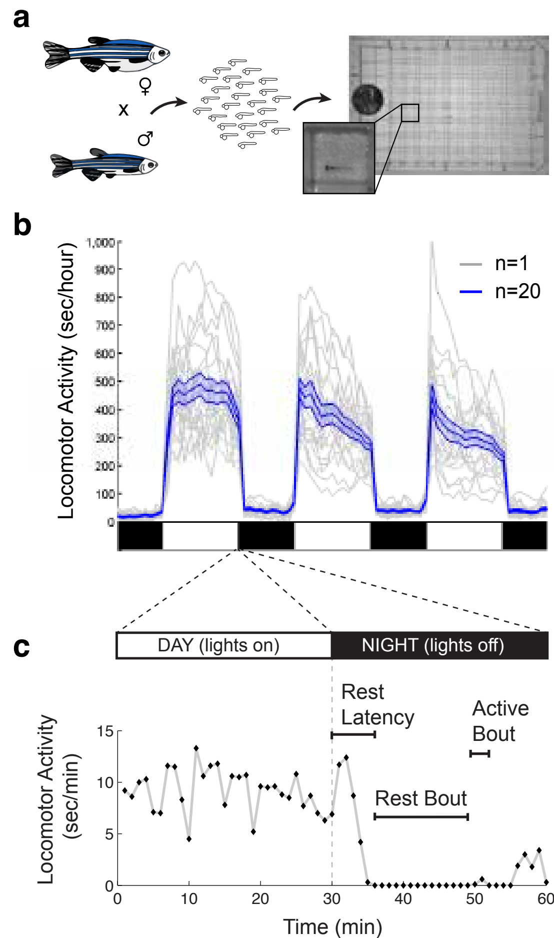

Figure 1.2: Larval zebrafish locomotor activity assay. Individual zebrafish larva are placed in each well of a 96-well-plate on the 4th day of development. The plate is placed in a temperature-controlled chamber that is illuminated by white lights during the day and is continuously illuminated by infrared lights. The larvae are monitored by an infrared camera and the locomotor activity of each larva is recorded by a computer. (B) Representative locomotor activity data for each of 20 individual wild-type larvae (gray traces) and their mean locomotor activity (blue trace, standard error of the mean) is shown. Black and white bars indicate day and night, respectively. Larvae are more active during the day than at night, although there is considerable variability among individuals. (C) An example of typical larval zebrafish behavior at the end of the day is shown. A rest bout is defined as a period of at least 1 min of inactivity, which is associated with an increase in arousal threshold (Prober et al., 2006). Rest latency indicates the time between lights off at night and initiation of the first rest bout.

Based on these criteria, behavioral sleep states have been demonstrated in flies and worms (Hendricks et al., 2000, Raizen et al., 2008, Shaw et al., 2000, Van Buskirk & Sternberg, 2007), and indeed, a number of studies have documented at least the first criterion for behavioral sleep in many fish species (Reebs, 1992). Recently, larval and adult zebrafish have been reported to exhibit all three behavioral criteria for sleep (Prober et al., 2006, Yokogawa et al., 2007, Zhdanova et al., 2001).

Criterion 1: quiescent state regulated by circadian rhythm

Starting around 4 days post fertilization (dpf), zebrafish raised on a 24 hour alternating light:dark cycle (e.g. 14h light:10h dark) exhibit daily fluctuations in locomotor activity (Hurd & Cahill, 2002, Prober et al., 2006). Like humans, zebrafish are diurnal and thus exhibit peak activity during the light phase and increased quiescence during the dark phase (Figure 1.2. Particularly at night, zebrafish spend bouts of several minutes or longer in a state of inactivity.

As has been observed in many other animals, larval and adult zebrafish that have been entrained on a light:dark cycle maintain circadian oscillations in locomotor activity even after external circadian cues are removed (Hurd & Cahill, 2002, Hurd et al., 1998). The core molecular machinery of the mammalian circadian clock is well conserved in zebrafish (Vatine et al., 2011, reviewed in), although zebrafish possess two paralogs of some mammalian genes (Postlethwait et al., 1998). A notable difference between zebrafish and mammals is that zebrafish peripheral circadian clocks are directly entrainable by light (Pando et al., 2001, Whitmore et al., 2000), a function that may have evolved in zebrafish due to their relative transparency. This inno-

vation suggests that zebrafish may not require a "master clock" analogous to the mammalian SCN to orchestrate circadian rhythms throughout the body. We also note that some widely-used inbred strains of laboratory mice lack enzymes required to synthesize melatonin (Ebihara et al., 1986, Goto et al., 2007), a hormone produced in the pineal gland which is thought to play a key role in transmitting circadian cues in humans and zebrafish. Thus, the role of the pineal gland and melatonin might be underestimated in mammalian research using these laboratory mouse strains. This fact, together with the diurnal sleep/wake pattern of zebrafish, suggests that zebrafish have some important advantages over rodents for modeling the circadian regulation of human sleep.

Criterion 2: reduced sensory responsiveness

Sleeping animals exhibit reduced responsiveness to sensory stimuli, which distinguishes sleep from quiet wakefulness. During quiescent periods, larval zebrafish show reduced responsiveness to mechanical stimuli (Zhdanova et al., 2001) and delayed responses to sudden changes in light intensity (Prober et al., 2006), and quiescent adult zebrafish are less responsive to electrical stimuli (Yokogawa et al., 2007). Because larval zebrafish exhibit reduced responsiveness after at least 1 minute of inactivity, sleep in larval zebrafish has been operationally defined as a quiescent bout lasting at least 1 minute. A similar approach has been used to define sleep in adult zebrafish as a minimum 6-second inactive bout. (Yokogawa et al., 2007). Whether the difference between adult and larval sleep is biological or methodological remains to be clarified. Nonetheless, this approach to defining sleep states has been useful for identifying

evolutionarily conserved sleep regulators in zebrafish (see next section). However, additional work may further refine the definition of zebrafish sleep by using detailed assays of physiology and arousal across various sensory modalities during quiescence. For example, one report indicates that quiescence during day and night are not equivalent, based on the observation that nighttime quiescence is associated with reduced respiration and postural changes compared to daytime quiescence (Zhdanova, 2006).

Criterion 3: homeostatic regulation

A common approach to assaying homeostatic regulation of sleep is to test whether compensatory sleep occurs following a period of deprivation. Indeed, both larval and adult zebrafish exhibit this so-called "sleep rebound" behavior. In a study of larval zebrafish (Zhdanova et al., 2001), a vibration stimulus applied during the last 6 hours of the night resulted in sleep rebound the following day. The reduced locomotor activity during sleep rebound was accompanied by a significantly decreased sensitivity to a mechanical stimulus as compared to siblings not subjected to sleep deprivation. In a study of adult zebrafish (Yokogawa et al., 2007), electroshock or light stimuli applied for 6 hours at night reduced locomotor activity the following day, although arousal threshold was not assessed. Notably, sleep rebound in zebrafish has only been observed in dark testing conditions, whereas light appears to be a potent arousal stimulus that masks the effect of sleep deprivation (Yokogawa et al., 2007).

Although these data are suggestive of rebound sleep, further advances in both technique and knowledge are needed to firmly establish homeostatic control of sleep in zebrafish. One important consideration in the design of sleep deprivation studies

is the possibility of off-target effects of the deprivation protocol. For example, while light is a profoundly arousing stimulus for zebrafish, its utility as a specific sleep deprivation stimulus is limited because light also affects the circadian clock. Another confounding effect of sleep deprivation that can vary with different deprivation protocols is stimulus-induced stress, which may be caused by the prolonged and high-amplitude stimulus application needed to overcome behavioral habituation and sleep. The use of yoked test subjects that are stimulated randomly relative to sleep bouts is an important control for stress effects. However, data using this methodology have thus far only yielded modest effects of sleep deprivation on sleep rebound (Yokogawa et al., 2007).

Technical issues aside, an important, unresolved scientific issue is whether the amount of sleep rebound is proportionate to the amount of sleep deprivation in zebrafish. Additionally, better-refined definitions of zebrafish sleep and more sophisticated methods of monitoring and quantifying sleep states will provide new possibilities to study whether sleep deprivation affects sleep quality. For example, it would be interesting to test the hypothesis that sleep deprivation increases the depth in addition to the duration of sleep rebound in zebrafish.

1.2.2 Zebrafish arousal states

Whereas sleep and waking are relatively easy to define with objective behavioral criteria, specific arousal states are more difficult to characterize. An animal's arousal state can be characterized by: 1) changes in frequency or intensity of voluntary

locomotor activity, and 2) altered responsiveness to sensory or emotional stimuli (Pfaff et al., 2008). In addition to these general characteristics, arousal can be characterized by the specific behavioral outputs that it motivates, such as reward-seeking and sexual or courtship behaviors.

Arousal-associated changes in locomotor activity can be triggered by intense stimuli. For example, in response to sudden changes in light intensity (e.g. light to darkness over 10ms), larval zebrafish exhibit a biphasic response that begins with a transient, high-amplitude movement followed by a sustained, low-amplitude increase in locomotor activity that persists for at least several minutes (Emran et al., 2010, Prober et al., 2006). In addition to external stimuli, arousal states are also triggered by physiological drives such as hunger, sex, and pain. For example, adult zebrafish respond to caloric restriction with the same bi-phasic behavioral response resulting from mammalian hunger; starved fish are initially hyperactive, but become lethargic after prolonged caloric restriction (Novak et al., 2005). Arousal states can also be manifested as goal-seeking behaviors that change the structure of spontaneous locomotor activity. Food-seeking behavior is readily measured in larval zebrafish, which begin to hunt for food almost as soon as they can swim. This behavior can be quantitatively described by a temporal sequence that begins with ocular angle convergence followed by a series of orienting "J-turns" and forward swimming towards the target (Bianco et al., 2011, Borla et al., 2002, Gahtan et al., 2005, McElligott & O'Malley, 2005). These eye and tail movements are distinct from routine, spontaneous movements, enabling the objective identification of a food-seeking arousal state in both

free-swimming and partially restrained preparations.

Zebrafish exhibiting heightened locomotor activity can also exhibit enhanced sensory responsiveness, consistent with the behavioral criteria for arousal. For example, zebrafish exposed to a sudden change in water flow rate become hyperactive and respond more quickly to a repeat application of the flow stimulus (Yokogawa et al., 2012). Zebrafish also exhibit similarly enhanced responses to a whole-field visual motion stimulus, which is thought to be a crucial sensory cue underlying the behavioral response to water flow. Notably, flow-induced arousal did not affect responses to electroshock and touch stimuli, which is suggestive of distinct arousal states. Although this study provides evidence of sensory modality-specific arousal states, the time-courses and behavioral readouts of the other stimuli were substantially different from the flow-related stimuli, leaving open the interesting question of what determines the specificity of an arousal state.

1.3 Sleep/arousal neuromodulatory systems in zebrafish

The neuroanatomical and neurochemical systems that regulate sleep and arousal in mammals are largely conserved in zebrafish (Figure 1.1). One notable difference is that zebrafish lack midbrain dopaminergic neurons analogous to the mammalian vPAG and VTA (Holzschuh et al., 2001, Kaslin & Panula, 2001, McLean & Fetcho, 2004, Rink & Wullimann, 2002), although the less-studied mammalian dopaminergic

A11 group, which is noted for its roles in sensorimotor function and the human sleep disorder restless legs syndrome (Mignot et al., 2002), has a likely homolog in zebrafish ventral diencephalic dopamine clusters (Ryu et al., 2007, Tay et al., 2011). Also, zebrafish do not have a layered cortex, a principal target of mammalian ascending arousal systems, although homologies between mammalian cortical areas and zones in the zebrafish dorsal telencephalon have been proposed based on common molecular developmental patterns (Wullimann & Mueller, 2004). Basal forebrain and brainstem cholinergic neurons have not been clearly described in zebrafish larvae.

Importantly, the neuropharmacology of mammalian behavior is well-conserved in zebrafish (Rihel & Schier, 2012), and drugs or bioactive agents that affect sleep and/or arousal in mammals produce comparable effects in zebrafish. Zebrafish exhibit a dose-dependent decrease in locomotor activity when treated with known hyponotics and sedatives, including melatonin, GABA receptor agonists (e.g. benzodiazepines, barbiturates, diazepam), histamine H1 receptor antagonists, and $\alpha 2$ adrenergic receptor agonists (Renier et al., 2007, Ruuskanen et al., 2005, Sundvik & Panula, 2012, Zhdanova et al., 2001). More recently, an unbiased screen of nearly 4000 small molecules corroborated the roles of arousal and sleep modulators, including noradrenaline, serotonin, dopamine, GABA, glutamate, histamine, adenosine and melatonin, in regulating zebrafish sleep/wake behavior (Rihel et al., 2010). Studies examining more specialized aspects of arousal, such as sensorimotor responses, have also confirmed the role of key monaminergic systems, including dopamine and serotonin, in regulating arousal states in zebrafish (Burgess & Granato, 2007, Mu et al., 2012, Yokogawa et al., 2012).

Conserved Hypocretin system in zebrafish

The hypocretin (Hcrt) neuromodulatory system is the best-characterized regulator of sleep and arousal in zebrafish, and we focus on these studies to illustrate the current discoveries, concepts and issues that arise when studying neuromodulators of zebrafish sleep and arousal.

The zebrafish *hcrt* gene encodes two structurally-related peptides homologous to mammalian Hcrt1 and Hcrt2 (Kaslin et al., 2004, Faraco et al., 2006). Among vertebrates, including zebrafish, there is particularly high sequence homology near the C-terminus of each Hcrt peptide, which is the critical region for biological activity and receptor selectivity (Asahi et al., 1999, Darker et al., 2001, Lang et al., 2004). The zebrafish genome contains a single *hcrt receptor* ortholog (*hcrtr2*; previously named *hcrtr*), a G-protein coupled receptor (GPCR) that is structurally similar to the two mammalian *hcrt receptor* paralogs (Prober et al., 2006, Yokogawa et al., 2007).

At 5 dpf, when larval zebrafish sleep/wake behaviors are first observed, *hcrt* is specifically expressed in a bilateral nucleus in the posterior hypothalamus that encompasses ∼10 neurons per hemisphere, as determined by *in situ* hybridization (ISH) and immunohistochemistry using a Hcrt1-specific antibody (Faraco et al., 2006, Prober et al., 2006). Furthermore, enhanced green fluorescent protein (EGFP) expression driven by various *hcrt* upstream promotor sequences faithfully recapitulates the endogenous *hcrt* expression pattern (Faraco et al., 2006, Prober et al., 2006) (Figure 1.3). This cluster expands to approximately 40 neurons in the adult zebrafish hypothalamus (Appelbaum et al., 2009, Kaslin et al., 2004, Appelbaum et al., 2010). While

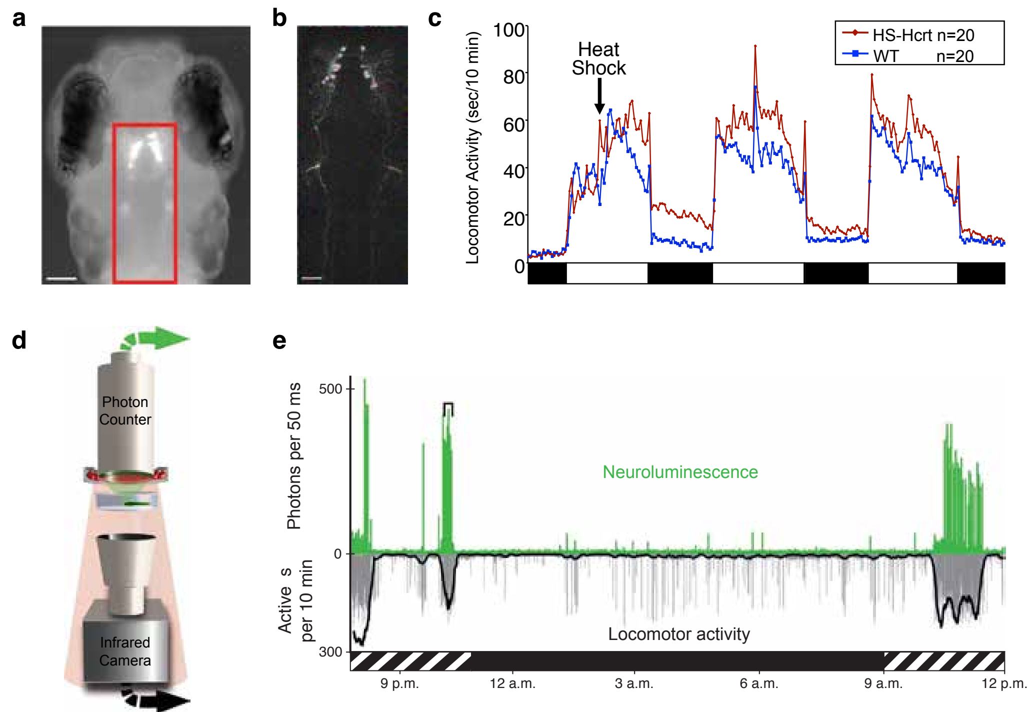

Figure 1.3: Hypocretin's arousal-promoting role is conserved in zebrafish (A) Dorsal view of a 4 dpf zebrafish larva that expresses GFP-Aequorin (GA) specifically in Hcrt neurons. (B) Two-photon z-projection image of the boxed area in (A). Scale bars represent 100μm (A) and 50μm (B). (C) Overexpression of Hcrt using a heat shock-inducible promoter (HS-Hcrt) increases locomotor activity. The mean locomotor activity of 20 HS-Hcrt larvae and 20 of their wild-type siblings is shown. The spike in activity during the afternoon of the 2nd and 3rd days of the experiment resulted from the addition of water to offset evaporation. (D) The GA assay. A large-area photon-counting photomultiplier tube is placed above a transparent behavior chamber in which a zebrafish larva that expresses GA in specific neurons is allowed to freely swim. The larva is imaged using infrared (IR) lights and an IR camera that is placed below the recording chamber. Spectral separation between GA neuroluminescence and the IR illumination allows the simultaneous recording of GA neuroluminescence and larval behavior. (E) Activity of Hcrt neurons during natural behavior. Data for a representative 4 dpf larva is shown. The larva exhibited periods of increased spontaneous locomotor activity (lower trace, thick line indicates 10min running average) during the subjective day (hatched bar below graph) and little activity during the subjective night (black bar below graph). Most neuroluminescence signals produced by Hcrt neurons (upper trace) coincide with periods of robust locomotor activity during the subjective day, suggesting that Hcrt neuron activity is associated with arousal.

the zebrafish *hcrt* expression pattern is consistent with mammals, the number of *hcrt* neurons is on the order of 102 fewer in zebrafish (Prober et al., 2006, Sakurai et al., 1998, de Lecea et al., 1998, Lin et al., 1999, Peyron et al., 2000). Two molecular markers of mammalian *hcrt* neurons, vesicular glutamate transporter and neuronal pentraxin2, also colocalize with *hcrt* in zebrafish (Appelbaum et al., 2009, 2010). Thus, the zebrafish provides a simple vertebrate system to study the development and function of Hcrt neurons.

Zebrafish *hcrt* neurons send widespread ascending and descending projections to brain areas associated with arousal (Figure 1.3), as they do in mammals (Peyron et al., 1998, Taheri et al., 1999). Using a transgenic *hcrt*:EGFP line, Hcrt projections in larval zebrafish were found in close apposition to noradrenergic cells of the locus coeruleus and processes of diencephalic dopaminergic cells in larval zebrafish (Prober et al., 2006). By adulthood, Hcrt-immunoreactive fibers contact these targets, and also densely innervate the serotonergic raphe and possibly histaminergic and cholinergic populations as well (Kaslin et al., 2004).

Consistent with widespread Hcrt neuron projections and with mammalian *hcrtr* expression, zebrafish *hcrtr2* expression is considerably more extensive than *hcrt* and is detected in widespread areas of the zebrafish brain. In larval zebrafish, one study used a high-resolution double fluorescent ISH method and found that *hcrtr2* colocalizes with dopamine beta hydroxylase in noradrenergic cells of the locus coeruleus and dopamine transporter in diencephalic dopaminergic cells (Prober et al., 2006), as in mammals (Trivedi et al., 1998, Marcus et al., 2001). However, another study con-

cluded that there is no *hcrtr2* coexpression with these monoaminergic populations in 2 dpf or adult zebrafish, and instead reported that *hcrtr2* is expressed in GABAergic, adrenergic, cholinergic, and glycinergic systems (Yokogawa et al., 2007). The discrepancies reported in these studies may stem from differences in detection method, probe sensitivity and specificity, and possibly the developmental stages studied. For example, the latter study made extensive use of a two-color chromogenic ISH procedure, which cannot reliably report colocalization because the differently colored stains can mask each other, the colors cannot be spectrally separated into distinct channels, and high resolution imaging methods such as confocal microscopy cannot be used to resolve chromogenic stained samples in three dimensions (Jowett & Yan, 1996, Vize et al., 2009, Lauter et al., 2011). Neither study observed expression of *hcrtr2* in histaminergic or serotonergic cells. However, the methods used in both studies, especially fluorescent ISH, are often not sensitive enough to detect low-abundance transcripts such as those encoding GPCRs, so negative data obtained using this method should be interpreted with caution. Indeed, a close examination of data obtained by the latter study suggests coexpression of faintly stained *hcrtr2* in some monoaminergic nuclei, which is consistent with the former study.

Several studies have also explored the functional role of the *hcrt* system in zebrafish. One study of larval zebrafish found that heat shock inducible overexpression of a *hcrt* transgene promotes wakefulness by consolidating active states, increasing arousal, and reducing sleep (Prober et al., 2006). Thus, the zebrafish Hcrt gain of-function (GOF) phenotype (Figure 1.3) is comparable to the effects of intracere-

broventricular injection of Hcrt peptide in rodents (Thakkar et al., 2001, España et al., 2001) and goldfish (Nakamachi et al., 2006). Conversely, sleep fragmentation is observed in adult zebrafish containing a null mutation in the *hcrtr2* gene (Yokogawa et al., 2007). This loss-of-function (LOF) result is strikingly similar to the sleep/wake fragmentation observed in rodents, canines, and humans that lack Hcrt signaling (Sutcliffe & de Lecea, 2002). However, this study also made a controversial proposal that the zebrafish Hcrt system may be functionally divergent from the mammalian Hcrt system based on the observation that the *hcrtr2* mutant displayed a mild decrease in sleep, whereas Hcrt peptide injection caused a mild decrease in locomotor activity. However, the decreased sleep in *hcrtr2* mutants was only significant compared to unrelated, non-mutagenized animals, and the decreased locomotor activity following Hcrt peptide injection may have resulted from the relatively high doses of peptide used (280-2800 pmol/g body weight in adult zebrafish versus 2.8-28 pmol/g body weight that increased locomotor activity in adult goldfish). Indeed, a subsequent study using the same *hcrtr2* mutant did not report a decreased sleep phenotype (Appelbaum et al., 2009). More recently, this debate seems to have been resolved by a report that inducible ablation of *hcrt* neurons using a genetically targeted toxin increased sleep and sleep/wake transitions in larval zebrafish (Elbaz et al., 2012). Thus, in non-invasive, inducible zebrafish systems, Hcrt GOF consolidates sleep/wake states and reduces sleep, whereas Hcrt LOF fragments sleep/wake states and increases sleep.

Taken together, the neuroanatomical and functional data from different research groups indicate that the zebrafish Hcrt system is interconnected with major neu-

romodulatory arousal systems, and regulates sleep/wake transitions by promoting arousal. Thus, the zebrafish provides a simple model of the vertebrate Hcrt system. The zebrafish system is poised to tackle unanswered questions about how Hcrt regulates sleep and arousal states. For example, although the zebrafish data point to a role for Hcrt in arousal state regulation, its specific role in different forms of arousal is unknown. Zebrafish can also be used to characterize the neural mechanisms through which Hcrt affects sleep and arousal using GOF and LOF genetic tools, such as heat shock inducible Hcrt transgenic zebrafish and the *hcrtr2* mutant, in combination with mutant and transgenic zebrafish that lack other neuromodulatory systems. The relatively small number of Hcrt neurons should also facilitate studies of their development and connectivity, as well as analysis of their activity during different behaviors. Finally, small molecule screens in zebrafish can be used to identify therapeutic pathways for the treatment of narcolepsy.

1.4 Outline of Thesis

Still in its infancy, zebrafish research on sleep and arousal has focused on establishing behavioral assays and identifying known regulators of behavioral states. Now that the zebrafish has been established as a useful model of vertebrate sleep and arousal, it is well suited among commonly used model organisms to address two long-standing yet fundamental questions in sleep research: first, what are the genetic mechanisms that regulate sleep and arousal behaviors, and second, what are the neural mechanisms that underlie these behaviors?

In subsequent chapters, I describe two studies that address the genetic and neural mechanisms that underlie sleep/arousal behavior. In Chapter 2, I introduce a large-scale genetic overexpression approach to screening that led to the discovery of a new regulator of sleep and arousal in the zebrafish. In follow up experiments, I uncover some of the candidate input and output pathways that govern the action of this neuropeptide. In Chapter 3, I introduce a new tool to manipulate neurons *in vivo*, and I demonstrate the feasibility of using this tool to study the neural mechanisms of sleep and arousal behavior in zebrafish. In Chapter 4, I discuss how these studies address and develop new hypotheses in the field. Additionally, I suggest future directions, and highlight ways in which the two present studies may be combined to further advance our understanding of sleep and arousal regulation.

Chapter 2

Discovery of a peptidergic neuromodulator of sleep/arousal behavior in zebrafish

Progress in understanding mechanisms that regulate mammalian sleep/arousal has been limited by the challenge of performing unbiased screens in mammals. This limitation is underscored by recent progress in understanding genetic and neuronal mechanisms that regulate *Drosophila* sleep through the use of screens (Cirelli et al., 2005, Koh et al., 2008, Pfeiffenberger & Allada, 2012, Rogulja & Young, 2012, Stavropoulos & Young, 2011). In contrast to mammals, zebrafish are uniquely well suited among vertebrate model systems for large-scale and unbiased 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).

However, there are several limitations in using forward genetics to identify genes that affect complex and quantitative behaviors such as sleep. First, as in mammals, sleep varies considerably among individual zebrafish, making it difficult to identify

a population of individuals that exhibit a recessive phenotype. Second, mapping genes responsible for recessive phenotypes requires a significant amount of time and labor, and it can be challenging for variable and quantitative phenotypes. Recent advances in deep sequencing technologies can accelerate this process (Miller et al., 2013, Obholzer et al., 2012), but they do not reduce the challenge of mapping a variable quantitative trait such as sleep. These two factors likely underlie the relative paucity of genes that have been identified by Drosophila forward genetic screens, despite large-scale efforts in several labs. Third, chemical mutagens typically induce thousands of mutations in each animal, and this high background mutational load may affect behavior. These problems can be avoided by performing insertional mutagenesis using retroviruses and transposons (Golling et al., 2002, Clark et al., 2011, Maddison et al., 2011, Trinh et al., 2011, Varshney et al., 2013), but these mutagens are much less efficient at inducing mutant phenotypes. This limitation is particularly problematic for screens that use relatively low-throughput assays, such as those required to identify sleep defects.

As an alternative to forward genetic approaches, reverse genetic techniques have undergone rapid growth over the last few years and promise to transform the use of zebrafish in the genetic analysis of behavior. One method, known as TILLING (Kettleborough et al., 2011) uses chemical mutagenesis to create thousands of mutant zebrafish, each of which is then screened for mutations in a gene of interest using deep sequencing. This approach is expected to identify null mutations in most zebrafish genes within the next few years, although it will likely fail to identify mutations

in many small genes. A second approach uses zinc finger nuclease (ZFN) and TAL-effector nuclease (TALEN) technologies (Bogdanove & Voytas, 2011). These nucleases can be designed to create double-stranded DNA breaks at specific sites in the genome, which are repaired by an error-prone process that often generates short insertions or deletions. A disadvantage of these approaches is that they preclude behavioral analysis of genes that are required for development.

In this study, we designed a new reverse-genetic screen approach to identify new molecular regulators of sleep/wake behavior by overcoming some of the major limitations of current screen approaches, and we discovered a novel peptide regulator of zebrafish sleep/wake behavior, Neuromedin U (Nmu). Using a combination of pharmacology and targeted mutation of Nmu receptors, we tested hypotheses about which molecular pathways mediate Nmu signaling in sleep/wake behavior. Furthermore, we performed histological assays to identify potential downstream pathways.

2.1 Methods

2.1.1 Generation of secretome library and microinjection assay

The LOCATE database (http://locate.imb.uq.edu.au) identifies approximately 4000 human proteins that are predicted or known to be secreted. Approximately 1600 open reading frames (ORFs) that encode a subset of these proteins are present in the hORFeome 3.1 collection, cloned in Gateway Entry vectors (Lamesch et al., 2007).

We constructed a Gateway Destination vector containing a heat shock-inducible promoter (Halloran et al., 2000) 5' to attR1 and an SV40 polyadenylation signal 3' to attR2, and the entire cassette was flanked by Tol2 transposase arms. LR clonase (Invitrogen) was used to transfer each ORF into the Destination vector. Plasmid preps were obtained using a Qiagen Biorobot 8000, purified using multiscreen PCR μ96 filter plates (LSKMPCR10, Millipore), dried using a speedvac, and dissolved in 10 μL water. Plasmid concentrations were measured using the Quant-iT kit (Invitrogen) and adjusted to 100 ng/μl. The purification step allowed injection of twice as much DNA compared to non-purified samples without increased toxicity. One nl of an injection mix (50 ng/μL plasmid, 150 ng/μL tol2 transposase mRNA and 5 mg/ml phenol red in 0.2 M KCl) was injected into the yolk of TLAB embryos at the one-cell stage. Each clone was injected into 50 embryos, and up to 32 larvae were tested in the behavioral assay. Injected animals were screened at 24 hpf and immediately prior to the behavioral assay to remove any larvae that exhibited abnormal morphology or locomotor behaviors. Clones for which fewer than 24 healthy larvae were available to test were reinjected at 25 ng/μL. All experiments with zebrafish followed standard protocols (Westerfield, 2000) in accordance with the Harvard University and California Institute of Technology Institutional Animal Care and Use Committee guidelines.

2.1.2 Secretome screen behavioral assay

Larval zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5°C, with lights on at 9 am and off at 11 pm. Individual larvae were placed into each of the

80 wells of a 96-well plate (7701-1651, Whatman) containing 650 μL embryo water (0.3 g/L Instant Ocean, 1 mg/L methylene blue, pH 7.0). Two Secretome clones were tested in each plate. At 4 days post-fertilization (dpf), larvae injected with each clone were loaded in alternating columns, and control larvae injected with the Destination vector lacking an ORF were loaded in columns 1 and 10. Locomotor activity was monitored for 72 hours using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch Monochrome camera (LTC0385, Bosch) fitted with a fixed-angle megapixel lens (M5018-MP, Computar) and infrared filter. Larvae were heat shocked at 37°C for one hour starting at 4 pm at 5 dpf. The movement of each larva was recorded using the quantization mode, and data from two cameras were collected in alternating minutes by one computer. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and illuminated with white lights from 9 am to 11 pm. 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 the following: detection threshold, 40; burst, 25; freeze, 4; bin size, 60 seconds. Data were processed using custom PERL and Matlab (The Mathworks, Inc) scripts. Clones that induced heat-shock dependent locomotor activity phenotypes were retested. Clones that produced the same phenotype upon retesting were used to generate stable transgenic lines using Tol2 transposase. Stable lines were identified by heat shocking the progeny of potential founders at 24 hours post-fertilization (hpf), fixing the embryos with 4% paraformaldehyde/phosphate buffered

saline (PBS) 1 hour after heat shock, and performing *in situ* hybridization using a probe specific for the overexpressed ORF. Lines that produced strong and ubiquitous gene overexpression were used for behavioral assays. Three independent stable lines were behaviorally tested for each ORF.

2.1.3 Generation of zebrafish heat-shock inducible transgenic lines

We used reciprocal BLAST to identify zebrafish orthologs for each human ORF that produced a locomotor activity phenotype in stable transgenic lines. To clone the zebrafish ORFs, we performed RT-PCR (Superscript III Reverse Transcriptase, Life Technologies) using mRNA isolated from 5 dpf zebrafish larvae (Trizol, Life Technologies). In cases where the 5' or 3' end of an ORF was not annotated in Ensembl or was ambiguous, 5' or 3' RACE (First Choice RLM-RACE Kit, Life Technologies) was performed to identify the entire ORF. Each ORF was cloned into a vector containing a heat-shock inducible promoter (Halloran et al., 2000) and ISce1 meganuclease sites, and each vector was injected with ISce1 (New England Biolabs) into zebrafish embryos at the one-cell stage to generate stable lines, which were identified as described above.

2.1.4 Generation of zebrafish mutants

nmu, *nmur1a*, and *nmur1b* mutant zebrafish were generated using zinc finger nucleases (ZFNs), as described (Chen et al., 2013). *nmur2* mutant zebrafish were generated

using TAL effector nucleases (TALENs), as described (Reyon et al., 2012). Plasmids were obtained from Addgene. *nmu* mutants were genotyped using the primers 5'-TGACCGACAGAGAGCATGAG-3', 5'-GGAGTAGTACCGCGAGCATC-3' and 5'-CGATTAAAACAGTAAAAACGCAGA-3', which generate 168 bp and 106 bp bands for the WT allele and a 164 bp band for the mutant allele. *nmur1* mutants were genotyped using the primers 5'-AGACACCCTGTATTTTCTCCTCA-3', 5'-GTAGAGGACGGGGTTTATGG-3' and 5'-CACATCGGAGCTAGCGAAAC-3', which generate a 203 bp band for the WT allele, and 214 bp and 96 bp bands for the mutant allele. *nmur1b* mutants were genotyped using the primers 5'-TCAAT GATACAGTACAACTGCTCCTC-3', 5'-AGGGTCCAAGGTATTTCTCCA-3' and 5'-ATGGTGCTCCACCAAAGAA-3', which generate 163 bp and 101 bp bands for the WT allele and a 155 bp band for the mutant allele. *nmur2* mutants were genotyped using the primers 5'-ATGACCGGGGTCTTAGGAAA-3' and 5'-TGACGTTT AACACGGAAGCA-3', which generate a 244 bp band for the WT allele and a 227 bp band for the mutant allele.

2.1.5 *in situ* hybridization

Zebrafish samples were fixed in 4% paraformaldehyde/PBS for 12-16 hours at room temperature. *in situ* hybridizations were performed using digoxygenin (DIG) labeled antisense riboprobes (DIG RNA Labeling Kit, Roche), as previously described (Thisse & Thisse, 2008). Double-fluorescent *in situ* hybridizations were performed using DIG- and 2,4-dinitrophenol (DNP)-labeled riboprobes and the TSA Plus DNP

System (PerkinElmer, Wellesley, MA). PCR products generated from larval zebrafish cDNA were used as templates for riboprobe synthesis using the following primers. *nmu*: 5'-CATGAGGAACAGCAATCAATG-3' and 5'-TAATACGACTCACTATAGG GACACATACTCATCAGATCTTCTTCC-3' (524 bp). *nmur1a*: 5'-GGCATTAAAC CTCACCGAGA-3' and 5'-TAATACGACTCACTATAGGGCACGTTTCGTCAAGAA ATCAAA-3' (1620 bp). *nmur1b*: 5'-GTGAACACGTCATGGTGCTC-3' and 5'-TAATACGACTCACTATAGGGGCGTTGGTATTCAGAAACTGC-3' (1206 bp). *nmur2*: 5'-CTCCTGACCTGCGCTGTAAT-3' and 5'-TAATACGACTCACTATAG GGGAGGAGCTGAACTTGACTTGC-3' (1486 bp). Plasmids containing *crh* (genbank clone CK352624, 849 bp) and *cfos* (genbank clone CA787334, 870 bp) expressed sequence tags were used for riboprobe synthesis.

2.1.6 Drug treatment

A concentrated stock of (R)-5'-(phenylaminocarbonylamino)spiro [1-azabicyclo [2.2.2]octane-3,2'(3'H)- furo [2,3-b]pyridine] (R-PSOP, AstraZeneca Neuroscience) was prepared in dimethyl sulfide (DMSO) just prior to the experiment. R-PSOP stocks were diluted into to final working concentration in standard embryo medium, E3. All conditions, including DMSO control, contained 0.05% DMSO. 4 dpf fish were treated with drug just prior to lights off before the commencement of the behavioral assay.

2.1.7 Statistical Analyses

In all statistical tests, the significance threshold was set to $p<0.05$. Asterisks in figures denote statistics for significant comparisons using Student's t-test or pairwise comparisons following ANOVA. Student's t-test was used for experimental designs containing 2 groups (i.e. HS-Nmu/+ versus WT comparisons, or 500μM RPSOP versus DMSO). 1-way ANOVA was performed for >2 comparisons (i.e. R-PSOP drug dosages and for Nmu mutants). 2-way ANOVA was performed for HS x Genotype experiments (i.e. HS-Nmu in receptor mutants). When the 2-way ANOVA revealed significant HSxGenotype interactions, further pairwise comparisons were performed using the Tukey-Kramer procedure for multiple comparisons, and significant pairwise comparisons are displayed on box plots. We did not observe any significant effects of Genotype factor alone, and for simplicity, these non-significant results are not displayed. "Day" box plots and statistics combine all daytime periods, and "Night" box plots and statistics combine nighttime periods displayed in the corresponding line graph. For heat-shock overexpression experiments, box plots and statistics were generated from post-heat shock timepoints; pre-heat shock data were not included in the box plot display and statistical analyses.

2.2 Results

2.2.1 A novel behavioral genetic ovexpression screen

Our genetic screen featured 4 key design components (Figure 2.1). First, the screen was conducted on a large-scale. From a publicly available database of human genes known or predicted to encode secreted proteins (a.k.a. Secretome), we selected ~1000 genes to test in the zebrafish. Second, we employed an inducible overexpression transgene cassette (HS-Sec, or Heat-Shock Secretome Gene). Induction of ubiquitous overexpression of a given Secretome candidate gene was achieved by incubating zebrafish larvae at 37°C for 1 hour. Third, we used a transgene injection approach for the primary screen. By directly testing fish injected with a HS-Sec transgene, we could circumvent the considerable labor, cost, and generation time involved with testing stable transgenic lines. Fourth, we employed a previously established high-throughput locomotor activity assay to identify any genes that, when over expressed, produced a quantifiable sleep/wake behavioral phenotype.

Screening was conducted in 2 phases. In Phase 1 of the screen, zebrafish single-cell embryos were injected with HS-Sec. In Phase 2, we re-tested each of the HS-Sec genes which produced a phenotype in Phase 1 using a stable transgenic line. In both phases of the screen, the phenotypic testing was performed by transferring 4 dpf zebrafish larvae to a high-throughput locomotor activity recording chamber, after which gene overexpression was induced on 5 dpf, and fish behavior was analyzed for altered sleep/wake phenotypes.

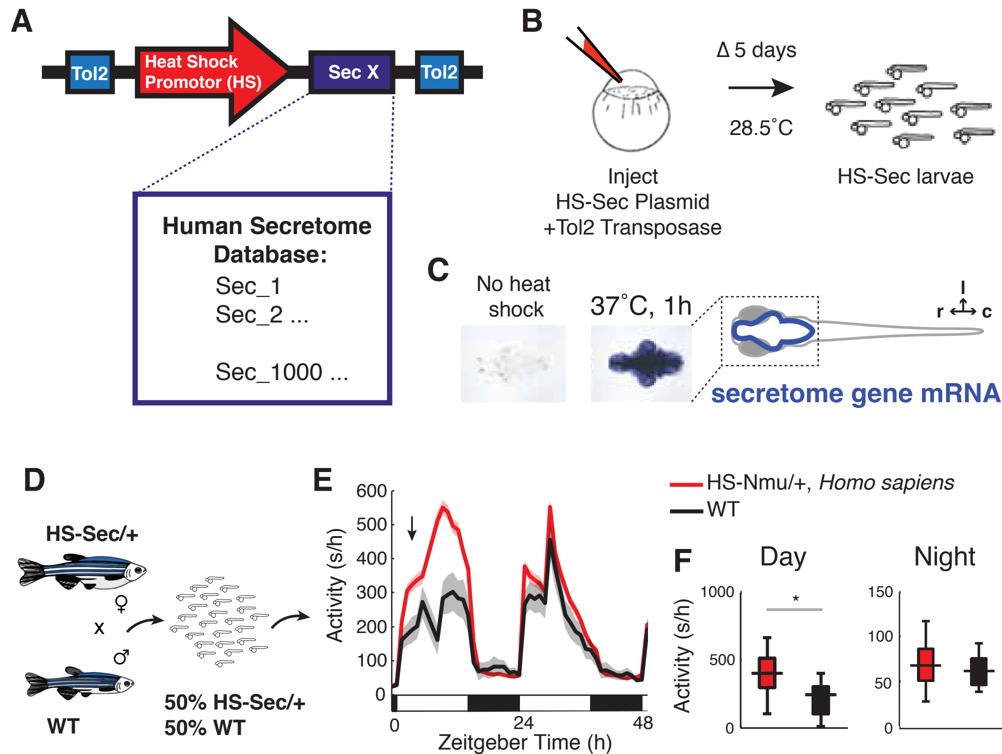

Figure 2.1: A behavioral genetic overexpression screen. A. The Heat Shock-Secretome (HS-Sec) overexpression transgenesis cassette. B. In Phase 1 of the screen, single cell zebrafish embryos are injected with the HS-Sec plasmid and Tol2 transposase mRNA to improve transgenesis efficiency (Kawakami et al., 2000). Prior to induction of gene overexpression, embryos are raised to 5 dpf at 28.5°C, a non-permissive temperature for the heat shock promotor. C. 1 hour, 37°C heat shock treatment induces expression of a Secretome gene in HS-Sec/+ larvae, as visualized by *in situ* hybridization analysis (purple stain) in dissected brain tissue. In the no-heat shock control (28.5°C), HS-Sec/+ larvae do not exhibit exogenous expression of the Secretome gene. The cartoon shows the approximate orientation of the dissected brain from a zebrafish larva. D. In Phase 2 of the screen, candidate Secretome genes were tested in stable transgenic zebrafish lines. E. Zebrafish larvae from a stable transgenic line possessing the HS-Sec transgene for a gene encoding human Neuromedin U (HS-Nmu/+, n=79) exhibited a significant increase in day time locomotor activity compared to simultaneously monitored wild type sibling controls (WT, n=43).
On all behavior figures in this chapter: Line plot values are mean ±S.E.M. White and black boxes on the x-axis denote daytime light periods and nighttime dark periods, respectively. Arrow indicates time of gene overexpression induction. Box plots indicate the median value (solid black line), 25th and 75th percentiles (box), and data range (whiskers). Parametric analysis of the data denoted by $*p<0.05$, $**p<0.001$. For details of calculations and statistical analyses, see Methods.

2.2.2 Discovery of Neuromedin U, a neuropeptidergic regulator of sleep/wake behavior

Overexpression of human Neuromedin U (HS-Nmu) produced the largest screen-wide locomotor phenotype. We observed that following a 1 hour, 37°C heat shock treatment to induce trangene expression, zebrafish larvae possessing the HS-Nmu (human) transgene exhibited significantly increased daytime locomotor activity and decreased sleep as compared to their wild-type sibling controls (Figure 2.1). Given that known mammalian neuromodulatory regulators of arousal are generally conserved in zebrafish (Chiu & Prober, 2013), we hypothesized that the zebrafish might also possess an endogenous Nmu system to regulate locomotor behavior.

To determine whether the mammalian Nmu system is conserved in zebrafish, we first cloned the zebrafish orthologue. We found that the zebrafish genome contains a single *nmu* orthologue that encodes a predicted Nmu mature peptide that is 56% identical to human Nmu-25 (Figure **??** and Figure 2.3). Based on structure-function studies of vertebrate Nmu, the C-terminal heptapeptide sequence contains the critical region for biological activity (Hashimoto et al., 1991, Sakura et al., 1991). We find that there is almost complete conservation of this region; zebrafish Nmu retains 6 of 7 human Nmu C-Terminal amino acids, including a Phe residue that is important for activity (Kurosawa et al., 1996) and an Arg residue that is indispensable for receptor binding and activation (Sakura et al., 2000).

Using *in situ* hybridization analysis, we found that zebrafish *nmu* is expressed in discrete nuclei within the central nervous system of 24-120 hpf zebrafish. At 24 hpf,

A

```
NMU_Dr    ATGAGGAACAGCAATCAATGTGAACGCGCAACCGCTCACAGCGCGATGAGCCCGGGAAACACCTCCGCCCTGATGCTCGC 80
NMU_Dr d4 ATGAGGAACAGCAATCAATGTGAACGCGCAACCGCTCACAGCGCGATGAGCCCGGGAAACACCTCCGCCCT----CTCGC 76

NMU_Dr    GGTACTACTCCTCTCCTTCATACCCATCACCACAAGTGCTCCGATGCTCTTGAATCCATCTTCACTAGAGCATGAGCAGC 160
NMU_Dr d4 GGTACTACTCCTCTCCTTCATACCCATCACCACAAGTGCTCCGATGCTCTTGAATCCATCTTCACTAGAGCATGAGCAGC 156

NMU_Dr    TACTAACCCAGATAACTGATTTGTGTTCATTCTACCTCTCCGCAGACCCGTCCTTTAGAACATCTGACGTCCTGGAGGAC 240
NMU_Dr d4 TACTAACCCAGATAACTGATTTGTGTTCATTCTACCTCTCCGCAGACCCGTCCTTTAGAACATCTGACGTCCTGGAGGAC 236

NMU_Dr    CTGTGTTTCCTAATGCTGGGATCACTGCAAAAATCGAAGGAGATCACAGCTCGAGAGACTAGCAAAAGGTTTTTATTTCA 320
NMU_Dr d4 CTGTGTTTCCTAATGCTGGGATCACTGCAAAAATCGAAGGAGATCACAGCTCGAGAGACTAGCAAAAGGTTTTTATTTCA 316

NMU_Dr    TTACACTAAACCAAACGGGGCAGGATTGTCTGATGGGACGTCTACTGTGTTGCACCCTCTTCTGGAGCTCATACCCCAGC 400
NMU_Dr d4 TTACACTAAACCAAACGGGGCAGGATTGTCTGATGGGACGTCTACTGTGTTGCACCCTCTTCTGGAGCTCATACCCCAGC 396

NMU_Dr    TTGCCAGAAGAAGAAGCAGGAGAATGAAATTAAATGAGAACCTTCAAGGTCCGGGACGCATCCAGAGCAGAGGATACTTC 480
NMU_Dr d4 TTGCCAGAAGAAGAAGCAGGAGAATGAAATTAAATGAGAACCTTCAAGGTCCGGGACGCATCCAGAGCAGAGGATACTTC 476

NMU_Dr    CTTTATCGGCCAAGAAATGGAAGAAGATCTGATGAGTATGTGTAA                                   525
NMU_Dr d4 CTTTATCGGCCAAGAAATGGAAGAAGATCTGATGAGTATGTGTAA                                   521
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B

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NMU_Hs    MLRTESCRPRSPAGQVAAA--SPLLLLLLLLAWCAGACRGAPIL-PQGLQPEQQLQLWNEIDDTCSSFLSIDSQPQASNALEELCFMIM 86
NMU_Mm    MSRAAGHRPGLSAGQLAAATASPLLSLLLLLACCADACKGVPIS-PQRLQPEQELQLWNEIHEACASFLSIDSRPQASVALRELCRIVM 88
NMU_Dr    MRNSNQCERATAHSAMSPGNTSALMLAVLLLSFIPITTSAPMLLNPSSLEHEQ---LLTQITDLCSFYLSADPSFRTSDVLEDLCFLML 86
NMU_Dr d4 MRNSNQCERATAHSAMSPGNTSALSRYYSSPSYPSPQVLRCS                                                42

NMU_Hs    GMLPKPQEQDEKDNTKRFLFHYSKTQKLGKSNVVSSVVHPLLQLVPHLHERRMKRFRVDEEFQSPFASQSRGYFLFRPRNGRRSAGFI 174
NMU_Mm    EISQKPQEQSEKDNTKRFLFHYSKTQKLGNSNVVSSVVHPLLQLVPQLHERRMKRFKA--EYQSPSVGQSKGYFLFRPRNGKRSTSFI 174
NMU_Dr    GSLQKSKEITARETSKRFLFHYTKPNGAGLSDGTSTVLHPLLELIPQLARRRSRRMKLNENLQGPGRIQSRGYFLYRPRNGRRSDEYV 174
NMU_Dr d4                                                                                           42
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C

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Nmur1a_Dr     ATGGAGATTGAAGACTTCTGCCTTGACCAAGACGAGTATCTGGAGAAATACCTCGGGCCAAGACGATCCCCAGTGTTCCTGCCTGTAT 88
Nmur1a_Dr i11 ATGGAGATTGAAGACTTCTGCCTTGACCAAGACGAGTATCTGGAGAAATACCTCGGGCCAAGACGATCCCCAGTGTTCCTGCCTGTAT 88

Nmur1a_Dr     GCCTGACCTACCTCCTGATCTTCCTGGTGGGAGCGGTGGGAAACATCCTTACCTGCATTGTCATTGCTAAAAACAAAGTCATGCGGAC 176
Nmur1a_Dr i11 GCCTGACCTACCTCCTGATCTTCCTGGTGGGAGCGGTGGGAAACATCCTTACCTGCATTGTCATTGCTAAAAACAAAGTCATGCGGAC 176

Nmur1a_Dr     GCCGACCAACTTCTACCTGTTCAGCCTGGCCATTTCAGATCTTCTAGTGCTTCTCCTGGGAATGCCTTTGGAGCTTTATGAAATGTGG 264
Nmur1a_Dr i11 GCCGACCAACTTCTACCTGTTCAGCCTGGCCATTTCAGATCTTCTAGTGCTTCTCCTGGGAATGCCTTTGGAGCTTTATGAAATGTGG 264

Nmur1a_Dr     AGCAACTATCCGTTCCTTTTAGGCAAGGGCGGTTGTTACTTCAAGACTCTTCTCTTCGAGACTGTTTGCTTTGCGTCGATCTTGAACG 352
Nmur1a_Dr i11 AGCAACTATCCGTTCCTTTTAGGCAAGGGCGGTTGTTACTTCAAGACTCTTCTCTTCGAGACTGTTTGCTTTGCGTCGATCTTGAACG 352

Nmur1a_Dr     TAACTGCTTTGAGCGTTGAACGCTACATTGCTGTGATTCACCCACTCCGAGCCAAATACGTAGTAACCCGCACTCATGCAAAGCGCCT 440
Nmur1a_Dr i11 TAACTGCTTTGAGCGTTGAACGCTACATTGCTGTGATTCACCCACTCCGAGCCAAATACGTAGTAACCCGCACTCATGCAAAGCGCCT 440

Nmur1a_Dr     GATATTGAGCGTCTGGAGCATTTCCGTGCTTTGCGCCATTCCCAACACGATCCTCCACGGTATATTTACTCTCCCGCCTCCTAAAGGG 528
Nmur1a_Dr i11 GATATTGAGCGTCTGGAGCATTTCCGTGCTTTGCGCCATTCCCAACACGATCCTCCACGGTATATTTACTCTCCCGCCTCCTAAAGGG 528

Nmur1a_Dr     AAAGCAGCAGGAGTCATGCTCGACTCCGCCACATGCATGCTCGTGAAACCGCGCTGGATGTACAACCTGATCATCCAAATCACGACTC 616
Nmur1a_Dr i11 AAAGCAGCAGGAGTCATGCTCGACTCCGCCACATGCATGCTCGTGAAACCGCGCTGGATGTACAACCTGATCATCCAAATCACGACTC 616

Nmur1a_Dr     TGCTATTCTTCCTGTTGCCCATGTTGACCATCAGCGTTTTGTATCTGCTCATCGGCATGCAGCTGAAGCGGGAGAAGATGCTGCAGGT 704
Nmur1a_Dr i11 TGCTATTCTTCCTGTTGCCCATGTTGACCATCAGCGTTTTGTATCTGCTCATCGGCATGCAGCTGAAGCGGGAGAAGATGCTGCAGGT 704

Nmur1a_Dr     CCTGGAGGCCAAAGCCAGTTCGGGCCTGGACAGCTCATCTAATGTGCGCAGTCAGCAGCAGAAAACCCGTCGCCAGCAGGTGACCAAG 792
Nmur1a_Dr i11 CCTGGAGGCCAAAGCCAGTTCGGGCCTGGACAGCTCATCTAATGTGCGCAGTCAGCAGCAGAAAACCCGTCGCCAGCAGGTGACCAAG 792

Nmur1a_Dr     ATGTTGTTTGTTTTGGTGGTGATGTTCGGCATCTGCTGGGCTCCGTTTCACACCGACCG-----------CCTGATGTGGAGCTTCAT 869
Nmur1a_Dr i11 ATGTTGTTTGTTTTGGTGGTGATGTTCGGCATCTGCTGGGCTCCGTTTCACACCGACCGGTTTCGCTAGCTCCGATGTGGAGCTTCAT 880

Nmur1a_Dr     GGATCAGAAGGACAGCGAGCACATCGAGATCTTTGAGGTCTACGAGTACGTCCACGTCATCTCCGGGGTCTTTTTCTACCTGAGCTCG 957
Nmur1a_Dr i11 GGATCAGAAGGACAGCGAGCACATCGAGATCTTTGAGGTCTACGAGTACGTCCACGTCATCTCCGGGGTCTTTTTCTACCTGAGCTCG 968

Nmur1a_Dr     GCCATAAACCCCGTCCTCTACAATCTGATGTCCACCCGCTTCAGGGAGATGTTCAAAGAGGTGATGTGCCACCATAAATGGCGTCCCG 1045
Nmur1a_Dr i11 GCCATAAACCCCGTCCTCTACAATCTGATGTCCACCCGCTTCAGGGAGATGTTCAAAGAGGTGATGTGCCACCATAAATGGCGTCCCG 1056

Nmur1a_Dr     TCCCGAGAAAGCGCTCTCTGAGCATGACCAGAGTCACCGTTCGCAGCACCGTCAGTGACGTCCCGCCATGCAACGGCACTGTGACTAT 1133
Nmur1a_Dr i11 TCCCGAGAAAGCGCTCTCTGAGCATGACCAGAGTCACCGTTCGCAGCACCGTCAGTGACGTCCCGCCATGCAACGGCACTGTGACTAT 1144

Nmur1a_Dr     TGAAGGAGACGATTATGACGTGGATGAAGGTCAAGAAAATAAGACATGTCCCTAA                                  1188
Nmur1a_Dr i11 TGAAGGAGACGATTATGACGTGGATGAAGGTCAAGAAAATAAGACATGTCCCTAA                                  1199
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Figure 2.2: Sequences of wild type and mutant NMU, Nmur1 and Nmur2. Nucleotide sequences of open reading frames of zebrafish wild type and mutant *nmu* (A, ENSDARG00000043299), *nmur1a* (C, ENSDARG00000060884), *nmur1b* (D, ENSDARG00000003944) and *nmur2* (F, ENSDARG00000022570) are shown. Zebrafish Nmur orthologs were identified by reciprocal Blast searches of mammalian and zebrafish genomes. Red boxes indicate ZFN (C, D) and TALEN (A, F) binding sites. Alignments of amino acid sequences of human, mouse, wild type zebrafish and mutant zebrafish NMU (B; human, ENSG00000109255; mouse, ENSMUSG00000029236), Nmur1 (E; human, ENSG00000171596; mouse, ENSMUSG00000026237) and Nmur2 (G; human, ENSG00000132911; mouse, ENSMUSG00000037393) are shown. Amino acids shaded in black are identical to human. Blue lines indicate mature human NMU peptide (B) and predicted human Nmur transmembrane domains (E, G). H. Phylogenetic tree of human, mouse and zebrafish Nmu receptors.

D

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Nmur1b_Dr    ATGATACAGTACAACTGCTCCTCTCCAAGTTCACTGTTCACAGTTGTTGTGAACACGTCATGGTGCTCCACCAAAGAAGAAGAATGT 87
Nmur1b_Dr d5 ATGATACAGTACAACTGCTCCTCTCCAAGTTCACTGTTCACAGTTGTTGTGAACACGTCATGGTGCTCCACCA-----GAAGAATGT 82

Nmur1b_Dr    CAAAACAGAACAGCAAACCTATCAGACCTCTGTCTATCCCGCAGTGCCTACCTGGAGAAATACCTTGGACCCTGTCGTTCACCTTTC 174
Nmur1b_Dr d5 CAAAACAGAACAGCAAACCTATCAGACCTCTGTCTATCCCGCAGTGCCTACCTGGAGAAATACCTTGGACCCTGTCGTTCACCTTTC 169

Nmur1b_Dr    TTTCTGCCTATATGTGTCACCTACCTGCTCATCTTTGTAGTAGGTGTGGTGGGCAACATCCTAACTTGTATTGTCATTACCCGTCAT 261
Nmur1b_Dr d5 TTTCTGCCTATATGTGTCACCTACCTGCTCATCTTTGTAGTAGGTGTGGTGGGCAACATCCTAACTTGTATTGTCATTACCCGTCAT 256

Nmur1b_Dr    CGCATCATGCGCACAACGACAAACTACTACCTGTTCAGCCTGGCCATTTCTGACCTCCTTGTGCTGTTGCTTGGCCTCCCGCTGGAG 348
Nmur1b_Dr d5 CGCATCATGCGCACAACGACAAACTACTACCTGTTCAGCCTGGCCATTTCTGACCTCCTTGTGCTGTTGCTTGGCCTCCCGCTGGAG 343

Nmur1b_Dr    CTTTATGAGCTCTGGAGCAACTATCCTTTTCTGTTTGGAATTTCTGGCTGCTACTTCAAAACCTGTCTCTTTGAGACAGTCTGCTTT 435
Nmur1b_Dr d5 CTTTATGAGCTCTGGAGCAACTATCCTTTTCTGTTTGGAATTTCTGGCTGCTACTTCAAAACCTGTCTCTTTGAGACAGTCTGCTTT 430

Nmur1b_Dr    GCCTCTGTTCTCAATGTTACAGCCTTAAGTGCAGAACGTTATAGAGCTATCATTCATCCTCTGCACACCAAACATGTTGCGACGAGT 522
Nmur1b_Dr d5 GCCTCTGTTCTCAATGTTACAGCCTTAAGTGCAGAACGTTATAGAGCTATCATTCATCCTCTGCACACCAAACATGTTGCGACGAGT 517

Nmur1b_Dr    GCTCATGCAAAGCGTGTTATCCTTGTGTTATGGGCTGTCTCTCTATTGTGTGCCTTGCCCAATACCAGTATTCATGGTGTGGAGATG 609
Nmur1b_Dr d5 GCTCATGCAAAGCGTGTTATCCTTGTGTTATGGGCTGTCTCTCTATTGTGTGCCTTGCCCAATACCAGTATTCATGGTGTGGAGATG 604

Nmur1b_Dr    TTAAAACCCCGTTTTGGACTTACTTTCCCAGAATCAGGTGTGTGTACAGTGGTACACGATAGGTGGATCTACAACCTGCTGGTGCAA 696
Nmur1b_Dr d5 TTAAAACCCCGTTTTGGACTTACTTTCCCAGAATCAGGTGTGTGTACAGTGGTACACGATAGGTGGATCTACAACCTGCTGGTGCAA 691

Nmur1b_Dr    GTGACAGCACTGCTGTTCTTCATACTGCCGATGCTGACTATCAGTGTGCTGTATGTGCTGATTGGCCTACAGCTGCACCGGGAAAGG 783
Nmur1b_Dr d5 GTGACAGCACTGCTGTTCTTCATACTGCCGATGCTGACTATCAGTGTGCTGTATGTGCTGATTGGCCTACAGCTGCACCGGGAAAGG 778

Nmur1b_Dr    GAGTGCTTTGATTCAAAGATCGTGCTCAATCAGGATGGGGTCAATCAGAGGGCACGTCATCGACAAGTCACAAAAATGCTTTGTGCA 870
Nmur1b_Dr d5 GAGTGCTTTGATTCAAAGATCGTGCTCAATCAGGATGGGGTCAATCAGAGGGCACGTCATCGACAAGTCACAAAAATGCTTTGTGCA 865

Nmur1b_Dr    TTGGTGATTGTGTTTGGAATCTGCTGGGCTCCCTTTCACATTGATCGTGTAATGTGGAGCTATATTGATGACTGGACTGCAGAGAAC 957
Nmur1b_Dr d5 TTGGTGATTGTGTTTGGAATCTGCTGGGCTCCCTTTCACATTGATCGTGTAATGTGGAGCTATATTGATGACTGGACTGCAGAGAAC 952

Nmur1b_Dr    CACCACATCTTTGAGTATGTGCATCTTCTATCTGGTGTCTTCTTCTACTTGAGCTCAGTGGTCAATCCCATCCTGTATAACCTCATG 1044
Nmur1b_Dr d5 CACCACATCTTTGAGTATGTGCATCTTCTATCTGGTGTCTTCTTCTACTTGAGCTCAGTGGTCAATCCCATCCTGTATAACCTCATG 1039

Nmur1b_Dr    TCTTCGCGCTTCAGAGAAATGTTCCGTGAGGTGGTGTGTCAAAAAGACCATCGTCAGCTCTCAATGAGTAGGGTCACATTACGTAGT 1131
Nmur1b_Dr d5 TCTTCGCGCTTCAGAGAAATGTTCCGTGAGGTGGTGTGTCAAAAAGACCATCGTCAGCTCTCAATGAGTAGGGTCACATTACGTAGT 1126

Nmur1b_Dr    GTTGTTTCTGCCTCTTTTTCTGCCTCCAATACAGTCTCATTTTCTGTAAAGTTCAACCCACGTGAGCTGCCACACACACTC       1212
Nmur1b_Dr d5 GTTGTTTCTGCCTCTTTTTCTGCCTCCAATACAGTCTCATTTTCTGTAAAGTTCAACCCACGTGAGCTGCCACACACACTC       1207
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E

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Nmur1_Hs      MTPLCLNCSVLPGDLYPGGARNPMACNGSAA---RGHFDPEDLNLTDEALRLKYLGPQQTELFMPICATYLLIFVVGAVGNGLTCLVI 85
Nmur1_Mm      MV-----------------------CNISEF---KWPYQPEDLNLTDEALRLKYLGPQQMKQFVPICVTYLLIFVVGTLGNGLTCTVI 62
Nmur1a_Dr     M-------------------------------------EIEDFCLDQDEYLEKYLGPRRSPVFLPVCLTYLLIFLVGAVGNILTCIVI 51
Nmur1a_Dr i11 M-------------------------------------EIEDFCLDQDEYLEKYLGPRRSPVFLPVCLTYLLIFLVGAVGNILTCIVI 51
Nmur1b_Dr     MIQY--NCSS-PSSLFTVVVNTSW-CSTKEEECQNRTANLSDLCLSRSAYLEKYLGPCRSPFFLPICVTYLLIFVVGVVGNILTCIVI 84
Nmur1b_Dr d5  MIQY--NCSS-PSSLFTVVVNTSW-CSTRRMSKQNSKPIRPLSIPQCLPGEIPWTLSFTFLSAYMCHLPAHLCSRCGGQHPNLYCHYP 84

Nmur1_Hs      LRHKAMRTPTNYYLFSLAVSDLLVLLVGLPLELYEMWHNYPFLLGVGGCYFRTLLFEMVCLASVLNVTALSVERYVAVVHPLQARSMV 173
Nmur1_Mm      LRNKTMRTPTNFYLFSLAVSDMLVLLVGLPLELYEMQQNYPFQLGASACYFRILLLETVCLASVLNVTALSVERYVAVVRPLQAKSVM 150
Nmur1a_Dr     AKNKVMRTPTNFYLFSLAISDLLVLLLGMPLELYEMWSNYPFLLGKGGCYFKTLLFETVCFASILNVTALSVERYIAVIHPLRAKYVV 139
Nmur1a_Dr i11 AKNKVMRTPTNFYLFSLAISDLLVLLLGMPLELYEMWSNYPFLLGKGGCYFKTLLFETVCFASILNVTALSVERYIAVIHPLRAKYVV 139
Nmur1b_Dr     TRHRIMRTTTNYYLFSLAISDLLVLLLGLPLELYELWSNYPFLFGISGCYFKTCLFETVCFASVLNVTALSAERYRAIIHPLHTKHVA 172
Nmur1b_Dr d5  SSHHAHNDKLLPVQPGHF                                                                       102

Nmur1_Hs      TRAHVRRVLGAVWGLAMLCSLPNTSLHGIRQLHVP---CRGPVPDSAVCMLVRPRALYNMVVQTTALLFFCLPMAIMSVLYLLIGLRL 258
Nmur1_Mm      TRAHVRRMVGAIWVLATLFSLPNTSLHGLSQLTVP---CRGPVPDSAICSLVGPMDFYKLVVLTTALLFFCLPMVTISVLYLLIGLRL 235
Nmur1a_Dr     TRTHAKRLILSVWSISVLCAIPNTILHGIFTLPPPKGKAAGVMLDSATCMLVKPRWMYNLIIQITTLLFFLLPMLTISVLYLLIGMQL 227
Nmur1a_Dr i11 TRTHAKRLILSVWSISVLCAIPNTILHGIFTLPPPKGKAAGVMLDSATCMLVKPRWMYNLIIQITTLLFFLLPMLTISVLYLLIGMQL 227
Nmur1b_Dr     TSAHAKRVILVLWAVSLLCALPNTSIHGVEMLKPRFGLT---FPESGVCTVVHDRWIYNLLVQVTALLFFILPMLTISVLYVLIGLQL 257
Nmur1b_Dr d5                                                                                           102

Nmur1_Hs      RRERLLLMQEAKGRGSAAARSRYTCRLQQHDRGRRQVTKMLFVLVVVFGICWAPFHADRVMWSVV-SQWTDGLHL--AFQHVHVISGI 343
Nmur1_Mm      RRERMLLQVEVKGRKTAATQETSHRRIQLQDRGRRQVTKMLFALVVVFGICWAPFHADRIMWSLVYGHSTEGLHL--AYQCVHIASGI 321
Nmur1a_Dr     KREKMLQVLEAKA--SSGLDSSSNVRSQQQKTRRQQVTKMLFVLVVMFGICWAPFHTDRLMWSFMDQKDSEHIEIFEVYEYVHVISGV 313
Nmur1a_Dr i11 KREKMLQVLEAKA--SSGLDSSSNVRSQQQKTRRQQVTKMLFVLVVMFGICWAPFHTDRFR                            286
Nmur1b_Dr     HRER--ECFDSKI--VLNQDGVN------QRARHRQVTKMLCALVIVFGICWAPFHIDRVMWSYIDDWTAENHHIFE---YVHLLSGV 332
Nmur1b_Dr d5                                                                                           102

Nmur1_Hs      FFYLGSAANPVLYSLMSSRFRETFQEALCLGA-CCHRLRPRHSSHSLSRMTTGSTLCDVGSLGSWVHPLAGN-DGPEAQQETDPS   426
Nmur1_Mm      FFYLGSAANPVLYSLMSTRFRETFLQALGLGTQCCHRRQPYHGSHNHIRLTTGSTLCDVGHRNSRDEPLAVN-EDPGCQQETDPS   405
Nmur1a_Dr     FFYLSSAINPVLYNLMSTRFREMFKEVMCHHK---WRPVPRKRSLSMTRVTVRSTVSDVPPCNGTVTIEGDDYDVDEGQENKTCP   395
Nmur1a_Dr i11                                                                                      286
Nmur1b_Dr     FFYLSSVVNPILYNLMSSRFREMFREVVCQKD---------HRQLSMSRVTLRSVVSASFSASNTVSF-SVKFNPRELPHTL      404
Nmur1b_Dr d5                                                                                       102
```

Figure 2.2 (continued)

F

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Nmur2_Dr     ATGATGGCCCTGATCTGCCTTTGCGAGTCTGTGAATACATCAGACCAGGATTATGAGTTCTGCAACAGCAGTACGTTCAACTTCACTGG 89
Nmur2_Dr d17 ATGATGGCCCTGATCTGCCTTTGCGAGTCTGTGAATACATCAGACCAGGATTATGAGTTCTGCAACAGCAGTACGTTCAACTTCACTGG 89

Nmur2_Dr     AAATGACAGTGCACACTGTTACCTTCAGACCATTGATGAAGTTCTGTTTAAGCTTCTGGGCCCAAGACGATCTCCGTTCTTCTTCCCTG 178
Nmur2_Dr d17 AAATGACAGTGCACACTGTTACCTTCAGACCATTGATGAAGTTCTGTTTAAGCTTCTGGGCCCAAGACGATCTCCGTTCTTCTTCCCTG 178

Nmur2_Dr     TAACCTGTACTTACATCCTCATCTTCATGACCGGGGTCTTAGGAAACCTCCTGACCTGCGCTGTAATCACTAAAGATCGAAAAATGCAA 267
Nmur2_Dr d17 TAACCTGTACTTACATCCTCATCTTCATGACCGGGGTCTTAGGAAACCTCCTGACCTGCGCTGTAATCACTAAAGATCGAAAAATGCAA 267

Nmur2_Dr     ACTCCCACCAACTTGTATCTCTTTAGCCTGGCCATCTCCGATCTTCTAGTGCTACTCTTCGGGATGCCTCTGGAAATCTACGAACTTTG 356
Nmur2_Dr d17 ACTCCCACCAACTTGTATCTC-----------------CGATCTTCTAGTGCTACTCTTCGGGATGCCTCTGGAAATCTACGAACTTTG 339

Nmur2_Dr     GCAAAACTACCCTTTTCCCTTCGGCGAGAGCATCTGCTGCTTTAAAATCTTCTTGTTCGAAACAGTTTGCTTTGCTTCCGTGTTAAACG 445
Nmur2_Dr d17 GCAAAACTACCCTTTTCCCTTCGGCGAGAGCATCTGCTGCTTTAAAATCTTCTTGTTCGAAACAGTTTGCTTTGCTTCCGTGTTAAACG 428

Nmur2_Dr     TCACAGTGCTAAGTGTGGAGCGATACATAGCTGTGATTCACCCGCTCAAAACCCGTTACGCCATCACCAACAAGCACGCTCGAAGGGTC 534
Nmur2_Dr d17 TCACAGTGCTAAGTGTGGAGCGATACATAGCTGTGATTCACCCGCTCAAAACCCGTTACGCCATCACCAACAAGCACGCTCGAAGGGTC 517

Nmur2_Dr     ATCGCTGGGGTTTGGGCTATGTCTCTGCTCTGCGCCGTCCCGAACACCTCCCTCCATGGCCTGCAGTATCAGTATCTGCCGGAGAGGGT 623
Nmur2_Dr d17 ATCGCTGGGGTTTGGGCTATGTCTCTGCTCTGCGCCGTCCCGAACACCTCCCTCCATGGCCTGCAGTATCAGTATCTGCCGGAGAGGGT 606

Nmur2_Dr     TCAGGAATCGGCTACCTGCAACCTGCTCAAGCCCAAATGGATGTACAACTTGGTGATCCAGATCACAACTGTGCTCTTCTACTTTGTTC 712
Nmur2_Dr d17 TCAGGAATCGGCTACCTGCAACCTGCTCAAGCCCAAATGGATGTACAACTTGGTGATCCAGATCACAACTGTGCTCTTCTACTTTGTTC 695

Nmur2_Dr     CCATGATGATGATCAGCGTGCTGTATCTGATGATCGGTCTGACGCTTGGCAGAGGGCAGAAGCAGAAAAAGGACAAGCTGGGAAGCAAT 801
Nmur2_Dr d17 CCATGATGATGATCAGCGTGCTGTATCTGATGATCGGTCTGACGCTTGGCAGAGGGCAGAAGCAGAAAAAGGACAAGCTGGGAAGCAAT 784

Nmur2_Dr     CACAGCAACGACAGCTGGAAAATCCATCTGGACAGCAGACGGAGAAGGCAGGTCACCAAGATGCACTTTGTGGTGGTATTAGTGTTTGC 890
Nmur2_Dr d17 CACAGCAACGACAGCTGGAAAATCCATCTGGACAGCAGACGGAGAAGGCAGGTCACCAAGATGCACTTTGTGGTGGTATTAGTGTTTGC 873

Nmur2_Dr     CATCTGCTGGGCTCCGTTCCACATCGACCGGCTCTTATGGAGCTTCATCACCAGTTGGACAGACCACATGCATAACATCTTTGAATATG 979
Nmur2_Dr d17 CATCTGCTGGGCTCCGTTCCACATCGACCGGCTCTTATGGAGCTTCATCACCAGTTGGACAGACCACATGCATAACATCTTTGAATATG 962

Nmur2_Dr     TGCACATAATCTCCGGCGTGCTCTTCTACCTCAGTTCAGCTGTAAACCCCATAATCTACAACCTGCTTTCCAGCCGCTTCCGGGAACGG 1068
Nmur2_Dr d17 TGCACATAATCTCCGGCGTGCTCTTCTACCTCAGTTCAGCTGTAAACCCCATAATCTACAACCTGCTTTCCAGCCGCTTCCGGGAACGG 1051

Nmur2_Dr     TTTCAAGCGCTGGTGTGCAGTCGCCTGTCAACTAGATCCTCACGTAATGATTCCATGCCTTTTTACATCATACCCAAAGACCCCCCGAC 1157
Nmur2_Dr d17 TTTCAAGCGCTGGTGTGCAGTCGCCTGTCAACTAGATCCTCACGTAATGATTCCATGCCTTTTTACATCATACCCAAAGACCCCCCGAC 1140

Nmur2_Dr     TGATTTCAAACGGACTGGA                                                                      1176
Nmur2_Dr d17 TGATTTCAAACGGACTGGA                                                                      1159
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G

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Nmur2_Hs     MSGM----EKLQNA--------SWIYQQKLEDPFQKHLNSTEEYLAFLCGPRRSHFFLPVSVVYVPIFVVGVIGNVLVCLVILQHQAMK 77
Nmur2_Mm     MG-------KLENA--------SWI-----HDSLMKYLNSTEEYLAYLCGPKRSDLSLPVSVVYALIFVVGVIGNLLVCLVIARHQTLK 69
Nmur2_Dr     MMALICLCESVNTSDQDYEFCNSSTFNFTGNDSAHCYLQTIDEVLFKLIGPRRSPFFFPVTCTYILIFMTGVLGNLITCAVITKDRKMQ 89
Nmur2_Dr d17 MMALICLCESVNTSDQDYEFCNSSTFNFTGNDSAHCYLQTIDEVLFKLIGPRRSPFFFPVTCTYILIFMTGVLGNLITCAVITKDRKMQ 89

Nmur2_Hs     TPTNYYLFSLAVSDLLVLLLGMPLEVYEMWRNYPFLFGPVGCYFKTALFETVCFASILSITTVSVERYVAILHPFRAKLQSTRRRALRI 166
Nmur2_Mm     TPTNYYLFSLAVSDLLVLLLGMPLEVYELWHNYPFLFGPVGCYFKTALFETVCFASILSVTTVSIERYVAIVHPFRAKLESTRRRALRI 158
Nmur2_Dr     TPTNLYLFSLAISDLLVLLFGMPLEIYELWQNYPFPFGESICCFKIFLFETVCFASVLNVTVLSVERYIAVIHPLKTRYAITNKHARRV 178
Nmur2_Dr d17 TPTNLYLRSSSATLRDASGNLRTLAKLPFSLRREHLLL                                                   127

Nmur2_Hs     LGIVWGFSVLFSLPNTSIHGIKFHYFPNGSLVPGSATCTVIKPMWIYNFIIQVTSFLFYLLPMTVISVLYYLMALRLKKDKSLEADEG- 254
Nmur2_Mm     LSLVWSFSVVFSLPNTSIHGIKFQQFPNGSSVPGSATCTVTKPIWVYNFIIQATSFLFYILPMTLISVLYYLMGLRLKRDESLEADKV- 246
Nmur2_Dr     IAGVWAMSLLCAVPNTSLHGLQYQYLPE--RVQESATCNLLKPKWMYNLVIQITTVLFYFVPMMMISVLYLMIGLTLGRGQKQKKDKLG 265
Nmur2_Dr d17                                                                                          127

Nmur2_Hs     --------NANIQRPCRKSVNKMLFVLVLVFAICWAPFHIDRLFFSFVEEWSESLAAVFNLVHVVSGVFFYLSSAVNPIIYNLLSRRFQ 335
Nmur2_Mm     --------TVNIHRPSRKSVTKMLFVLVLVFAICWTPFHVDRLFFSFVDEWTESLAAVFNLIHVVSGVFFYLSSAVNPIIYNLLSRRFR 327
Nmur2_Dr     SNHSNDSWKIHLDSRRRRQVTKMHFVVVLVFAICWAPFHIDRLLWSFITSWTDHMHNIFEYVHIISGVLFYLSSAVNPIIYNLLSSRFR 354
Nmur2_Dr d17                                                                                          127

Nmur2_Hs     AAFQNVISSFHKQW-HSQHDPQLPPAQRNIFLTECHFVELTEDIGPQFPCQSSMHNSHLPAALSSEQMSRTNYQSFHFNKT         415
Nmur2_Mm     AAFRNVVSPSCK-WCHEQHRPQGPPAQKVIFLTECHLVELTEDAGPQFPCQSSIHNTQLTTVPCVEEVP                     395
Nmur2_Dr     ERFQALVCSRLSTRS-SRNDS-MPFY--IIPK-----DPPT-DFKRTG                                          392
Nmur2_Dr d17                                                                                          127
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H

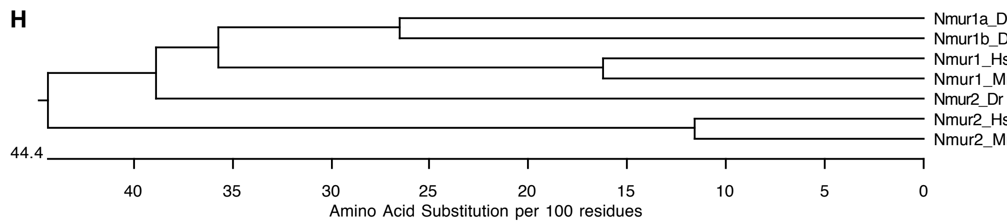

Figure 2.2 (continued)

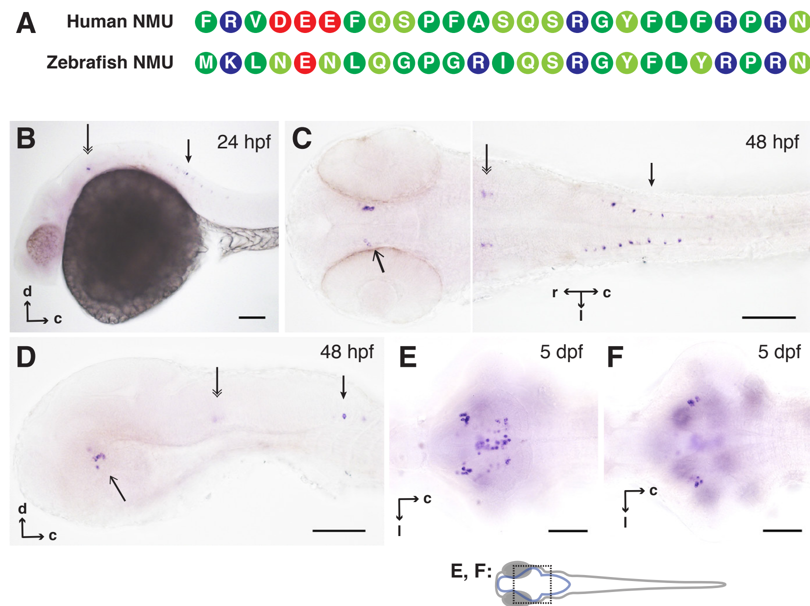

Figure 2.3: Nmu is conserved in zebrafish. A. The zebrafish predicted Nmu peptide sequence is well conserved from the human Nmu mature peptide. (Schematic modeled after (Malendowicz et al., 2012). Amino acids are color coded by type: blue=basic, red=acidic, green=neutral and non-polar, light green=neutral and polar). B-F. Endogenous expression of *nmu* transcript in the hypothalamus/pituitary (single arrow), brainstem (double arrow), and spinal cord (arrowhead) of 24-120 hpf zebrafish. At 24 hpf (B), hypothalamic *nmu* neurons are not yet specified. At 5dpf, zebrafish hypothalamic *nmu* expression differentiates into a few specific ventral (E) and more dorsal (F) cell clusters. Scale bars=100μm.

2 bilateral cell clusters occupy discrete regions of the brainstem and spinal cord, and these clusters persist at 48 and 120 hpf timepoints when additional cells are specified in the hypothalamus and pituitary. This pattern of expression in 120 hpf zebrafish larvae accords with tissue distribution of *nmu* in the adult mammalian central nervous system in pituitary, hypothalamus, brainstem, and spinal cord, but we did not observe peripheral expression of *nmu* in the gastrointestinal or genitourinary tracts, as has been reported in mammals (Austin et al., 1994, Howard et al., 2000, Szekeres et al., 2000, Fujii et al., 2000, Ivanov et al., 2002, Graham et al., 2003, Nogueiras et al., 2006).

We next tested whether Nmu function is evolutionarily conserved. We found that the locomotor phenotype of zebrafish larvae overexpressing the zebrafish orthologue recapitulated the increased locomotor activity phenotype induced by human Nmu overexpression in zebrafish (Figure 2.3 and Figure 2.1). Our observed phenotype concords with many mammalian studies in which various methods of Nmu administration results in increased locomotor activity and energy expenditure (Nakazato et al., 2000, Wren et al., 2002, Novak et al., 2006, 2007, Peier et al., 2009, Semjonous et al., 2009). Further analysis of zebrafish sleep and wake bout structure revealed that overexpression of Nmu increases wake bout length and latency to sleep while reciprocally decreasing sleep bout length and the number of sleep bout transitions. Thus, the overall effect of Nmu on sleep/wake architecture is to consolidate activity into periods of prolonged hyperactivity and insomnia in zebrafish (Figure 2.4).

The observed gain-of-function HS-Nmu phenotype predicts that a loss-of-function

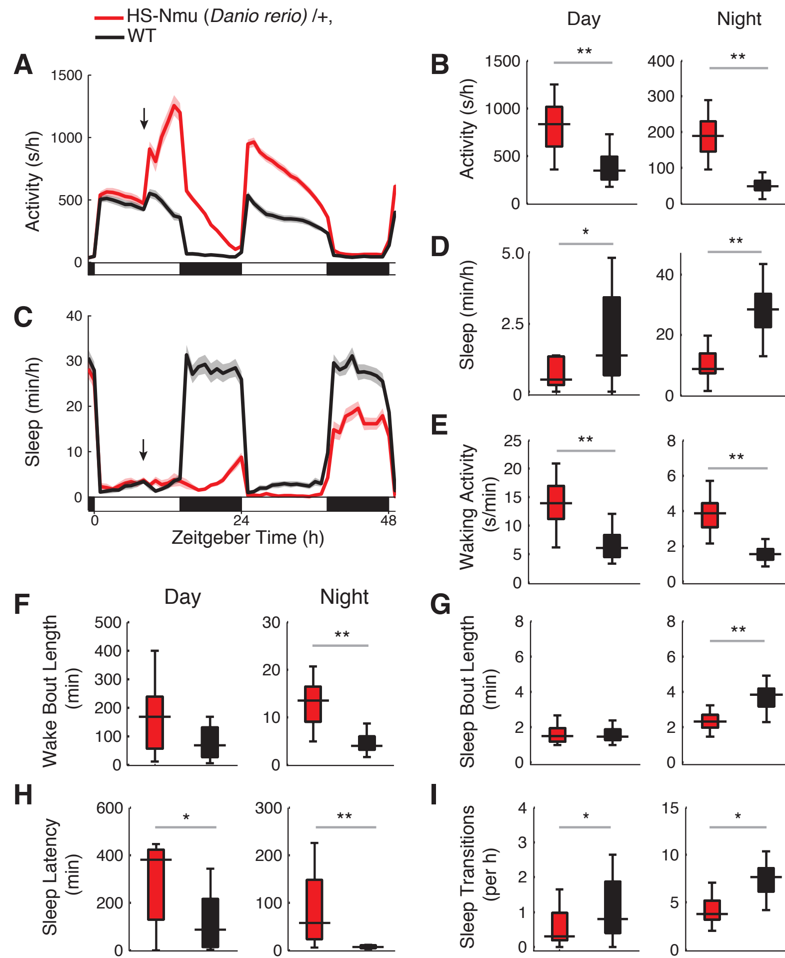

Figure 2.4: Nmu function is conserved in zebrafish A-D. Overexpression of zebrafish neuropeptide Nmu (HS-Nmu, n=44) induces locomotor activity and insomnia compared to simultaneously tested wild type siblings (WT, n=43). E-I. Sleep/wake architecture is altered in HS-Nmu zebrafish.

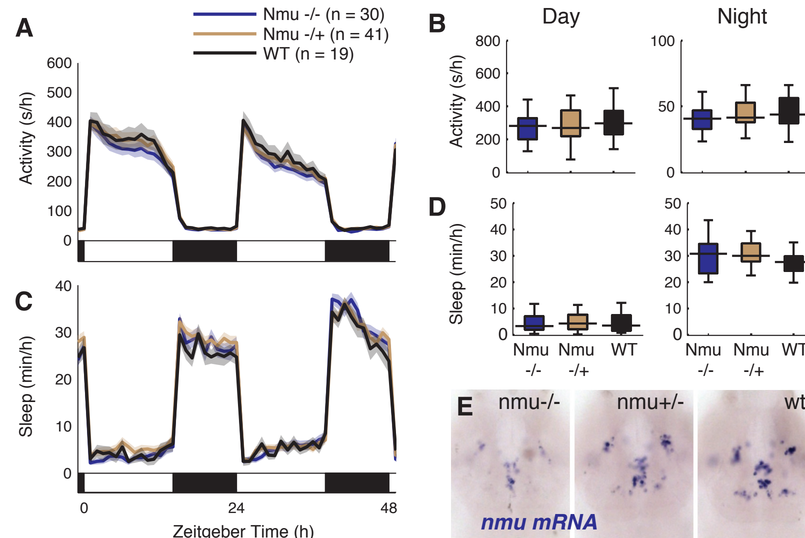

Figure 2.5: *nmu*$^{-/-}$ zebrafish exhibit normal locomotor activity and sleep. A-D. No significant differences among *nmu* mutants and wild type fish were observed. E. Reduced *nmu* transcript observed in the mutant is indicative of nonsense mediated mRNA decay.

manipulation of Nmu would result in hypoactivity. To test this, we generated a *nmu* mutant. This is predicted to be a null mutation because it produces a stop codon that should truncate the precursor upstream of the predicted Nmu peptide sequence (Figure 2.2). Furthermore, we observed evidence of nonsense-mediated mRNA decay (NMD) in *nmu*$^{-/-}$ fish by *in situ* hybridization analysis (Figure 2.5), suggesting that prematurely truncated transcripts are degraded before they can be translated into dominant negative or deleterious gain-of-function proteins (Chang et al., 2007). However, when we tested these mutants in our locomotor activity assay, we observed no differences in total locomotor activity or sleep among *nmu*$^{-/-}$, *nmu*$^{+/-}$, and *nmu*$^{+/+}$ siblings(Figure 2.5). This negative result suggests that other redundant mechanisms that regulate behavioral activity may compensate for the loss of *nmu* during development.

2.2.3 Nmur2 mediates the Nmu-induced sleep/wake phenotype

Next, we asked which Nmu receptors might be involved in regulating sleep/wake behavior. The zebrafish genome encodes 2 zebrafish Nmur1 orthologues, designated Nmur1a and Nmur1b, that are 52% and 49% identical to human Nmur1 (Figure 2.2), and encodes a single orthologue of Nmur2 that is 44% identical to human Nmur2. Using *in situ* hybridization analysis, we observed that *nmur2* expression is primarily expressed in discrete clusters in the zebrafish brain, including cell clusters in the brainstem, hypothalamus and forebrain (Figure 2.6).

We detected more restricted expression of nmur1a in a discrete cluster of hypothalmic cells, and we detected no *nmur1b* transcript in zebrafish up to 120 hpf. Thus, like mammals, zebrafish *nmur2* expression in CNS is more widespread compared to *nmur1* (reviewed by Brighton et al., 2004). Notably, we did not observe Nmu receptor expression in tissue outside of the CNS in 24, 48, or 120 hpf zebrafish, whereas in mammals, both *nmur1* and to a lesser extent *nmur2* are detected in many peripheral tissues, especially in cells of the gastrointestinal and genitourinary systems.

To test the functional requirement of each of these receptors in mediating the Nmu overexpression phenotype, we targeted a mutation to each of the 3 zebrafish Nmu receptor genes, *nmur1a*, *nmur1b*, and *nmur2* (Figure 2.2). For each of the 3 receptor mutants, homozygotes, heterozygotes, or wild-types (Genotype factor) were tested with and without the HS-Nmu transgene (HS-factor) (Figure 2.7 and Figure 2.8).

To determine whether the effects of HS-Nmu were dependent on receptor genotype,

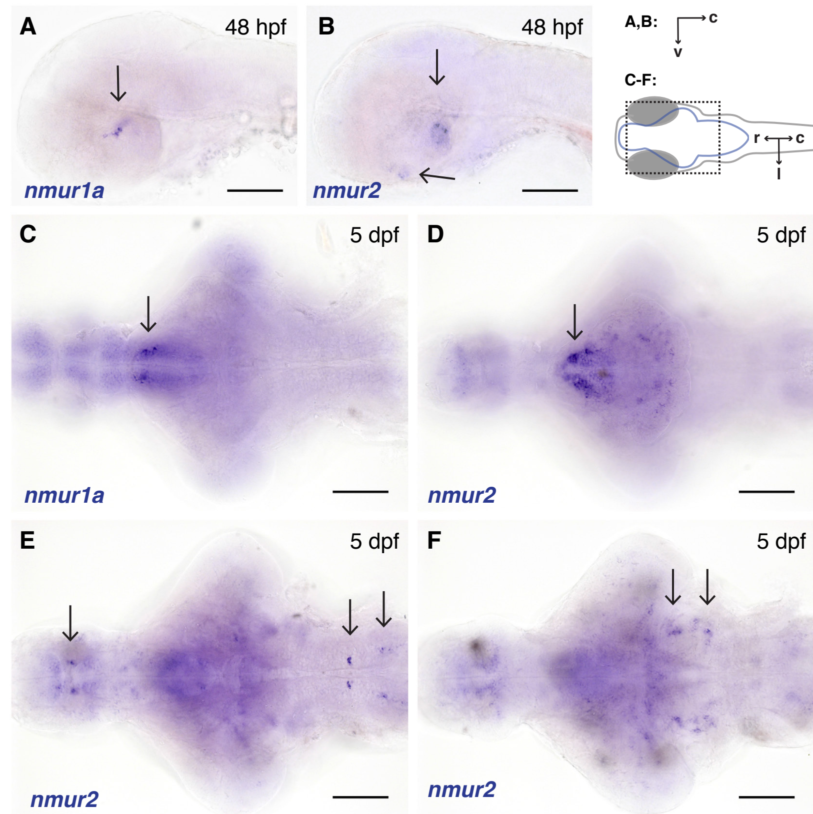

Figure 2.6: *in situ* hybridization analysis of Nmur gene transcripts in larval zebrafish. A-B. Lateral views of distribution of *nmur1a* and *nmur2* at 48 hpf. C. Highly restricted expression of *nmur1a* in 5 dpf zebrafish brain. D-F. Widespread but discrete expression of *nmur2* in 5 dpf zebrafish brain, starting from a ventral focal plane (D) and ending in a more dorsal focal plane (F). Scale bars=100μm.

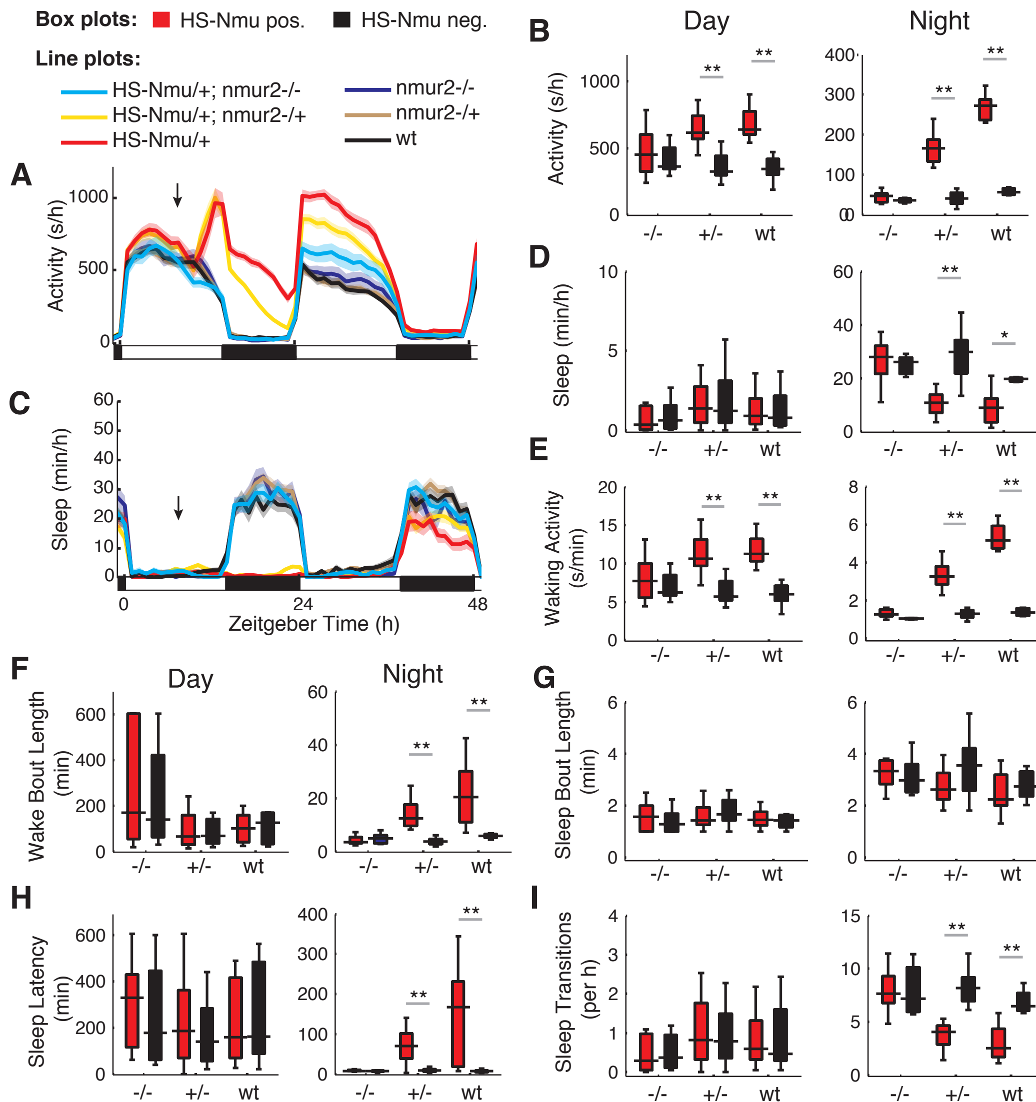

Figure 2.7: Nmu-induced hyperactivity and insomnia is mediated by *nmur2*. A-I. Zebrafish with a homozygous mutation of *nmur2* do not show significantly different sleep/wake phenotypes compared to wild type, and homozygous mutants do not respond to Nmu overexpression (compare HS-Nmu positive to HS-Nmu negative). In contrast, control siblings with heterozygous mutant or homozygous wild-type receptor alleles exhibit Nmu overexpression phenotypes. Number of subjects: HS-Nmu/+ *nmur2*$^{-/-}$ (n=24), HS-Nmu/+; *nmur2*$^{+/-}$ (n=42), HS-Nmu/+ (n=25), *nmur2*$^{-/-}$ (n=13), *nmur2*$^{+/-}$ (n=39), WT (n=23).

we tested for a significant interaction of Genotype and HS factors in each of the receptor mutants. We observed a significant interaction of *nmur2* Genotype x HS factors, whereas we did not observe significant interaction of *nmur1a* Genotype x HS factors or of *nmur1b* Genotype x HS factors. Importantly, post-hoc pairwise comparisons revealed significant effects of heat shock in *nmur2*$^{+/-}$ and in *nmur2*$^{+/+}$ fish, but no significant effect of heat shock in *nmur2*$^{-/-}$ fish. Thus, at least one wild-type allele of *nmur2* is required to permit HS-Nmu sleep/wake phenotypes, and one mutant allele of *nmur2* is sufficent to diminish the Nmu overexpression phenotypes. We found no significant main effect of receptor Genotype factor on observed locomotor activity or sleep, day or night, for any of the three mutated receptor genes, whereas the HS factor expectedly showed significant increases in locomotion in some of the pairwise comparisons. To summarize these experiments, we observed that the HS-Nmu sleep/wake phenotypes require *nmur2*, but not *nmur1a* or *nmur1b*.

Because we identified Nmur2 as a necessary mediator of HS-Nmu locomotor and sleep phenotypes, we hypothesized that blocking Nmur2 function would result in hypoactivity and altered sleep structure. Our lack of observed phenotypes in Nmu and receptor mutants might have been due developmental mechanisms, so rather than genetically blocking Nmur2 from the start of development, we acutely blocked Nmur2 function in 5 dpf wild-type zebrafish using a specific, pharmacological antagonist of Nmur2, R-PSOP (Liu et al., 2009). As predicted by our hypothesis, we observed that R-PSOP decreased locomotor activity and increased sleep in a dose-dependent manner (Figure 2.9). Notably, Nmur2 antagonism significantly increased transitions

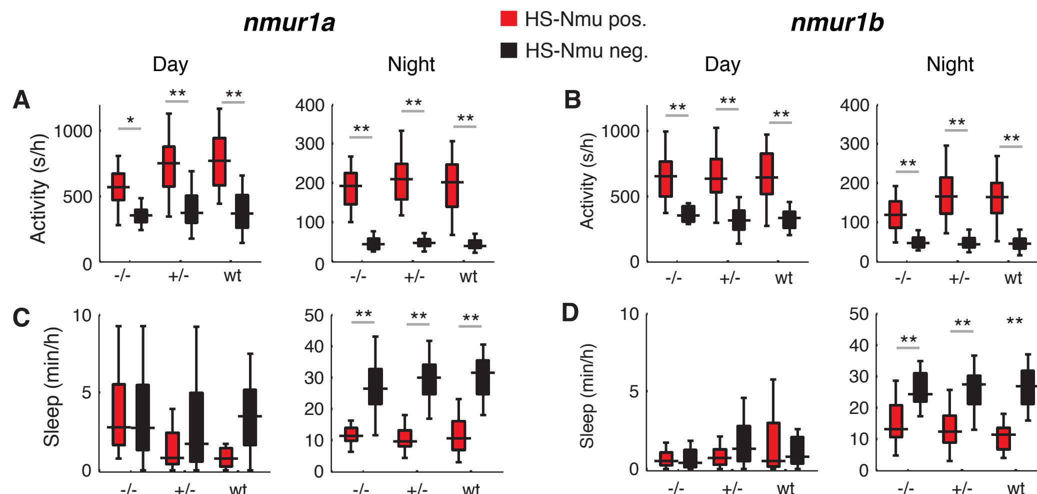

Figure 2.8: Nmu-induced hyperactivity and insomnia do not require *nmur1a* or *nmur1b*. Zebrafish with homozygous and heterozygous mutations of *nmur1a* (A,C) or *nmur1b*(B,D) respond to Nmu overexpression, indicating that these receptors are not required for HS-Nmu phenotypes. Number of subjects for *nmur1a* mutant experiment: HS-Nmu/+; *nmur1a*$^{-/-}$ (n=22), HS-Nmu/+; *nmur1a*$^{+/-}$ (n=9), HS-Nmu/+ (n=22), *nmur1a*$^{-/-}$ (n=23), *nmur1a*$^{+/-}$ (n=54), WT (n=18). Number of subjects for *nmur1b* mutant experiment: HS-Nmu/+; *nmur1b*$^{-/-}$ (n=29), HS-Nmu/+; *nmur1b*$^{+/-}$ (n=43), HS-Nmu/+ (n=25), *nmur1b*$^{-/-}$ (n=22), *nmur1b*$^{+/-}$ (n=39), WT (n=17).

to sleep, such that zebrafish larvae were highly prone to falling asleep during the day and night. This result concords with our Nmu overexpression data, and together, our results indicate that the function of endogenous Nmu is to promote and consolidate wakefulness.

2.2.4 Nmu sleep/wake phenotypes do not require glucocorticoid receptor

The that mechanisms link Nmu signaling via Nmur2 to a behavioral output are not known. A prominent model first proposed by Hanada et al. (2001) suggests that the Nmu locomotor phenotypes are a manifestation of the stress response that is mediated by the hypothalamic-pituitary-adrenal (HPA) axis. The classic molecular pathway of

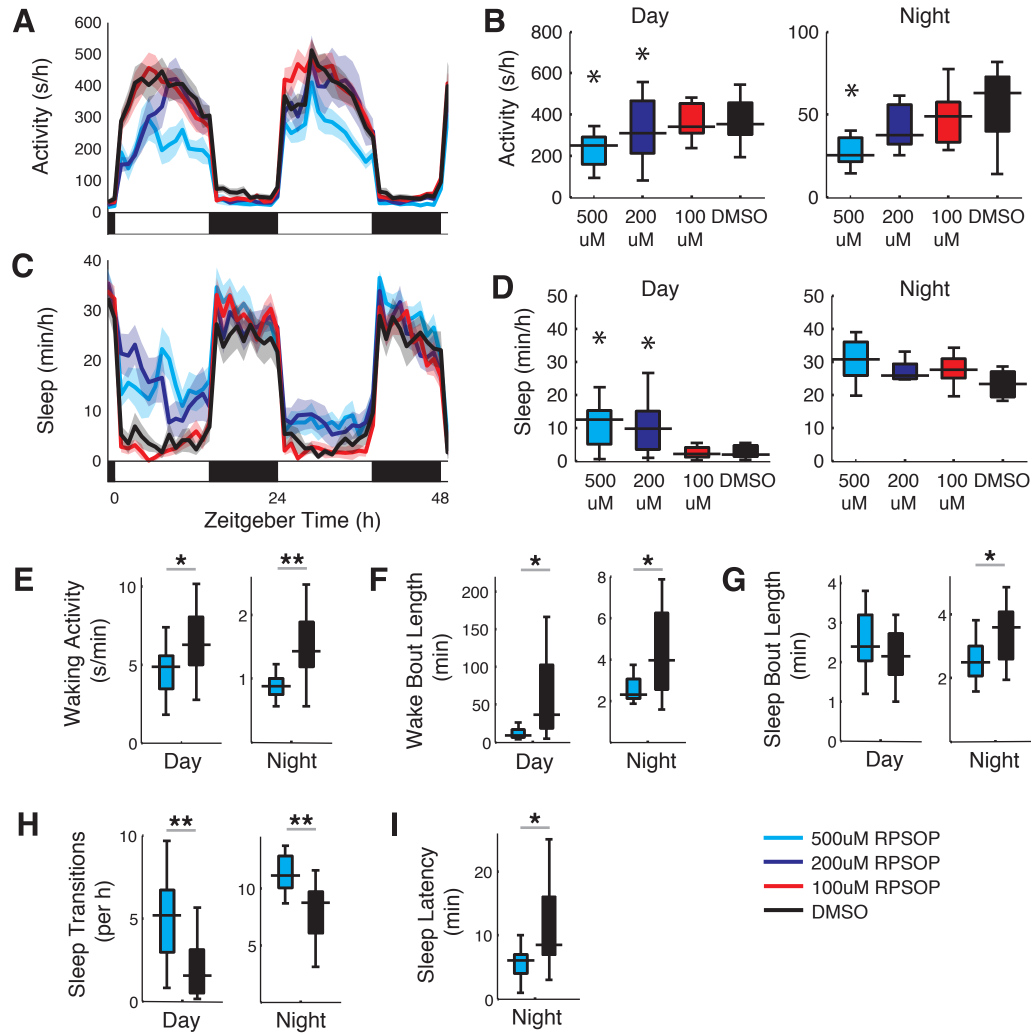

Figure 2.9: Dose-dependent effects of Nmur2 antagonism on sleep/wake architecture. A-D. Zebrafish treated with 200μM or 500μM concentrations of Nmur2 antagonist, R-PSOP, exhibit reduced locomotor activity and increased sleep compared to DMSO (vehicle) controls (n=12 per condition), whereas 100μM R-PSOP is not an effective dose. E-I. Zebrafish treated with 500μM R-PSOP show altered sleep/wake structure, with a notable increase in frequency of sleep transitions(H) during both day and night (n=24 per condition).

the HPA axis begins with signaling at corticotropin releasing hormone (Crh) neurons of the paraventricular nucleus (PVN) of the hypothalamus, and the signaling cascade eventually results in activation of adrenal cells that initiate glucocorticoid signaling. Several lines of evidence implicating the HPA axis in Nmu-mediated behaviors include: 1) *crh*$^{-/-}$ mice do not exhibit behavioral phenotypes that are caused by injection of Nmu peptide into the brain (Hanada et al., 2003); 2) Nmu injection was found to increase *cfos* expression in PVH (Ozaki et al., 2002, Ivanov et al., 2002); 3) among other regions, hypothalamic PVH contains Nmur2 cells (Raddatz et al., 2000); 4) Brain administration of Nmu increased plasma corticosterone levels in some, but not all, reports (Wren et al., 2002, Ozaki et al., 2002, Gartlon et al., 2004); 5) *crh*-positive neurons in the PVH are reduced in Nmu$^{-/-}$ mice (Hanada et al., 2004).

Based on predictions from mammalian data and generally well-conserved neuroendocrine systems in the larval zebrafish (Herget et al., 2014), we reasoned that the zebrafish would serve as a good model to test the hypothesis that the Nmu-induced hyperactivity phenotype requires glucocorticoid receptor, the classical target of the HPA output cascade. To test this, we analyzed the effect of Nmu overexpression in fish possessing a null mutation of the glucocorticoid receptor (*gr*$^{-/-}$) (Ziv et al., 2013). Surprisingly, the Nmu overexpression phenotype persists in the *gr*$^{-/-}$ mutant (Figure 2.10). This result strongly suggests that Nmu's behavioral effects are predominantly mediated by an alternative mechanism from the glucocorticoid receptor.

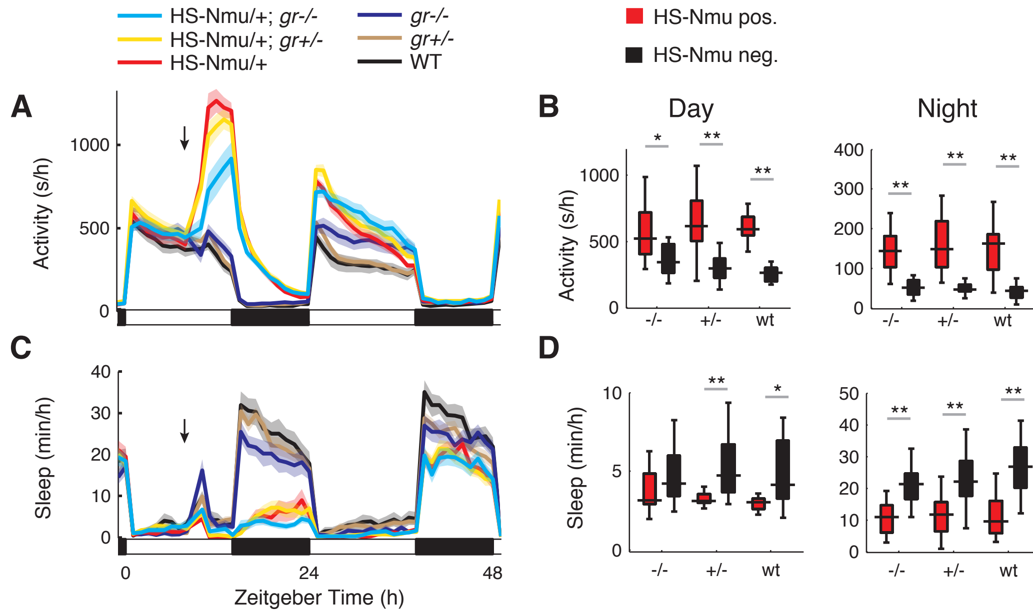

Figure 2.10: Nmu sleep/wake phenotypes do not require a key signaling component of HPA axis. A-D. Nmu overexpression phenotypes persists in $gr^{-/-}$ glucocorticoid receptor mutants. Additionally we did not observe significant differences in behavior between $gr^{-/-}$ and WT. Number of subjects: HS-Nmu/+; $gr^{-/-}$ (n=29), HS-Nmu/+; $gr^{+/-}$ (n=46), HS-Nmu/+ (n=29), $gr^{-/-}$ (n=21), $gr^{+/-}$ (n=44), WT (n=17).

2.2.5 Nmu-induced *cfos* activation in putative brainstem arousal systems containing *crh*

Our findings highlight the importance of examining alternative mechanisms by which Nmu might participate in sleep/wake regulation. We sought to generate new hypotheses by analyzing which cell populations are activated by Nmu overexpression. Expression of *cfos* or its protein product is widely used as an indicator of activated neurons, because ligand-binding to neurotransmitter receptors can lead to intracellular signaling cascades that cause transient *cfos* expression (Thompson et al., 1995). Indeed, *in vitro* numerous functional assays of human and rat recombinant Nmur2 show that the receptor can potently mediate intracellular calcium signaling (reviewed

by Brighton et al., 2004). Thus, we used *cfos* expression as a read-out for neurons that respond to Nmu signaling, both directly and indirectly through Nmu receptor signaling.

Using a *cfos* riboprobe, we found that Nmu overexpression activated several distributed cell populations, particularly in the forebrain and in discrete brainstem areas (Figure 2.11). Notably, a few clusters of the *cfos*-positive neurons mapped closely to *nmur2*-positive neurons in the brainstem. We observed comparatively little *cfos* expression in brains of non-transgenic siblings that underwent identical heat shock treatment, suggesting that the *cfos* labeling observed in HS-Nmu transgenics is specific to Nmu overexpression and not heat shock treatment.

The approximate anatomical location of the *cfos*-positive neurons suggested that they might belong to brainstem populations that promote arousal in vertebrates. In addition to hypothalamic populations of Crh-positive neurons, in human brains, Crh-positive neurons have been detected in brainstem arousal nuclei, including the locus coeruleus, parabrachial nucleus, and pedunculopontine tegmentum. Thus, we hypothesized that Nmu overexpression might activate some of these brainstem Crh expressing neurons in zebrafish. To test this, we performed double fluorescent ISH on brains from HS-Nmu fish and found that the brainstem *cfos*-positive neurons colocalized with *crh* (Figure 2.11). Interestingly, colocalization of *cfos* and *crh* was specific to the brainstem, whereas the hypothalamic *crh*-positive neurons, which are homologous to mammalian PVH Crh-expressing neurons of the HPA axis (Herget et al., 2014), do not colocalize with *cfos*. This result is consistent with our above

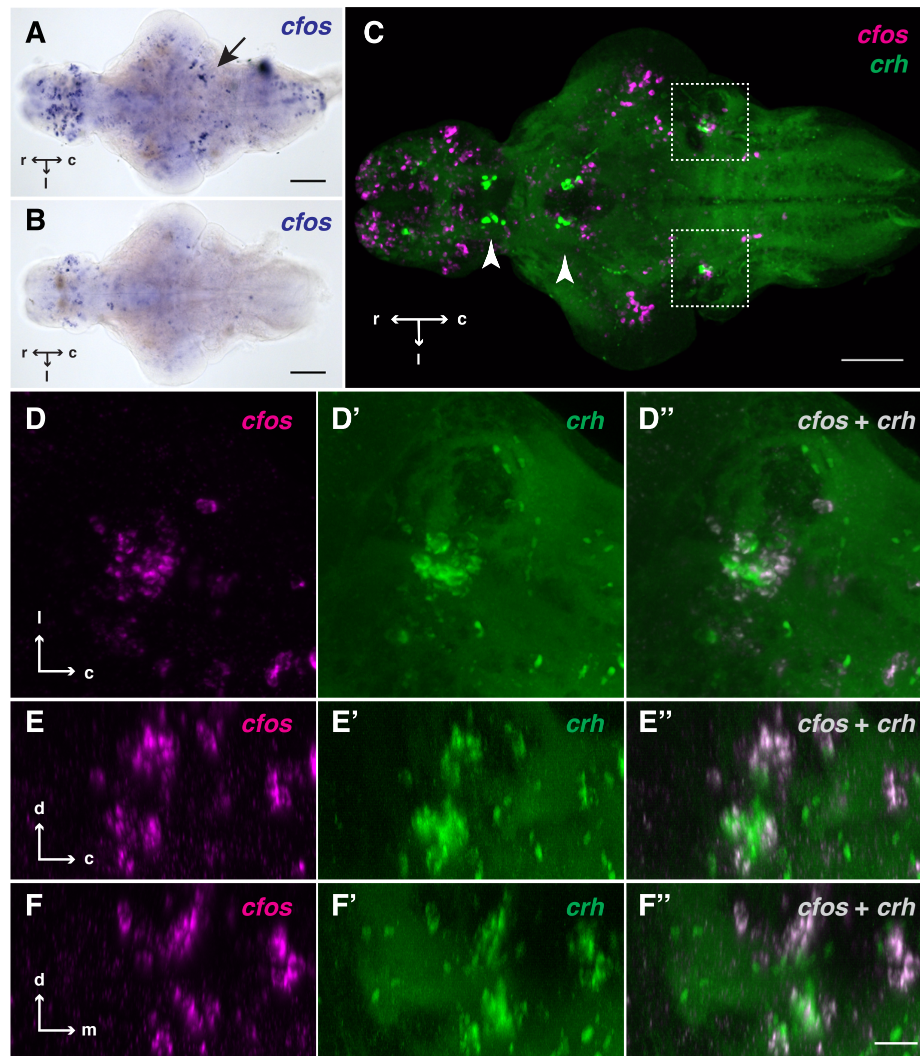

Figure 2.11: Nmu overexpression activates brainstem *crh* neurons. A. *in situ* hybridization analysis of *cfos* expression 1 hour after heat-shock induction of Nmu overexpression in a 5 dpf HS-Nmu/+ transgenic zebrafish brain. B. In the control condition, the same heat shock treatment does not elicit the same degree of *cfos* expression in wild-type fish. C-F. Double fluorescent *in situ* hybridization analysis reveals brainstem colocalization of *cfos* and *crh* expression following Nmu overexpression. A dorsal view of the entire brain (C) shows brainstem populations of *crh* that appear to colocalize with *cfos* (dotted boxes; upper box imaged at higher magnification in D-F), whereas diencephalic *crh* expression does not colocalize with *cfos* (arrowheads). The brainstem populations are presented at higher magnification (D), and in orthogonal views (E,F) to demonstrate colocalization. Scale bars=100um (A-C) and 20um(D-F).

glucocorticoid receptor mutant experiment, and provides further evidence that the HPA axis is not a critical mediator of Nmu signaling in arousal behavior.

2.3 Discussion

By combining injection-based gene overexpression with high-throughput locomotor activity assays, we developed a novel approach to identifying molecular regulators of zebrafish sleep/wake behavior.

Our design overcomes some typical screen limitations by combining several key features:

- no mutant/transgenic stable lines, which reduces labor and generation time
- no mapping of gene mutation to behavior, which reduces labor
- no background mutations, which improves chances of phenotype reproducibility
- heat shock inducible, which circumvents phenotype masking by developmental compensation
- uses over expressed secreted proteins, which can produce more robust phenotypes compared to haplo-insufficient mutagenesis or tissue-restrictive approaches
- uses library of >1000 human genes that encode secreted proteins, which reduces bias compared to a small-scale candidate approach

The collection of inducible overexpression plasmids generated in this study provides a resource library for the zebrafish research community that can be used to

identify secreted factors that regulate other behaviors or developmental processes in zebrafish. Furthermore, our approach could be extended in future studies to identify additional novel regulators of sleep/wake, for example, by screening for behavioral phenotypes in fish that over express unannotated open reading frames in the zebrafish genome.

Our screen identified human Neuromedin U as a candidate regulator of locomotor activity in the zebrafish. Our results show that this basic locomotor function and the key molecular features of the Nmu system are conserved from mammals to zebrafish. We further characterized a role for Nmu and its receptor Nmur2 in regulating sleep/wake architecture in zebrafish. Additionally, our molecular studies of downstream signaling pathways undermine the idea that Nmu-induced behavioral phenotypes are manifested as a classical HPA axis-mediated stress response. Instead, we propose here that Nmu's role in sleep/wake regulation might be mediated by extra-hypothalmic Crh neurons that activate brainstem arousal systems (Figure 2.12).

2.3.1 Conserved molecular circuitry and function of Nmu

We found that the gene, expression pattern in the brain, and peptide sequence of Nmu are well-conserved from mammals to zebrafish. Furthermore, we found that Nmu overexpression promotes wakefulness and hyperarousal in zebrafish. Compared to the HS-(human)Nmu transgenic, the HS-(zebrafish)Nmu transgenic exhibited a larger, more prolonged phenotype. However, it is unclear whether this reflects experimental variation (note differences in average wild-type activity levels between experiments)

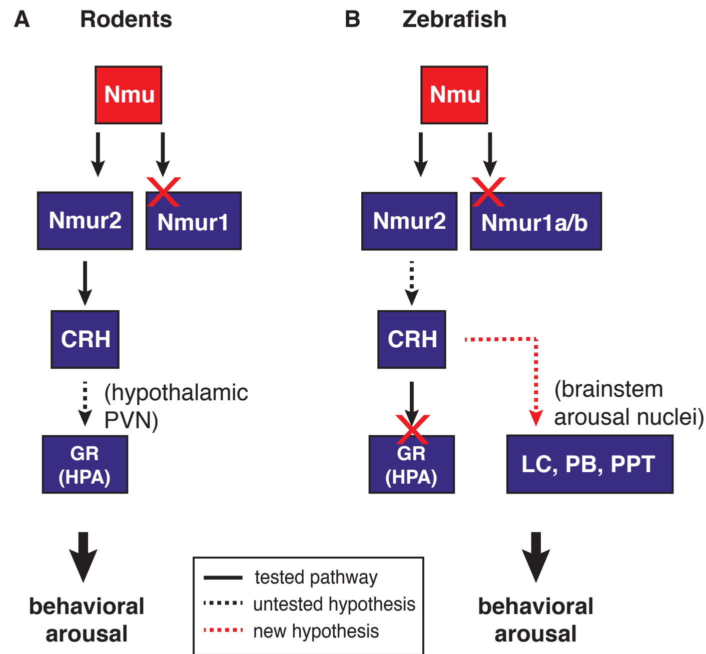

Figure 2.12: Newly tested and revised hypotheses of Nmu signaling in sleep/wake behavior. A. Rodent studies propose that Nmu-induced behavioral phenotypes are mediated by the Crh neurons of the paraventricular hypothalamus (PVH). PVH is the starting point of the hypothalamic-pituitary axis (HPA), in which glucocorticoids participate as the final effector molecule of the HPA signaling cascade. B. Our experiments in zebrafish demonstrate that Nmu's actions are mediated independently of the glucocorticoid receptor (GR), and put forth the hypothesis that sleep/wake behaviors are mediated by extra-hypothalamic sources of Crh that participate in brainstem arousal systems.

or differences in dosage or potency of the human versus zebrafish genes. Nonetheless, our data are completely consistent with the increased physical activity observed in mammals following acute administration of Nmu peptide (Nakazato et al., 2000, Wren et al., 2002, Novak et al., 2006, 2007, Peier et al., 2009, Semjonous et al., 2009).

The receptor circuitry is also conserved in zebrafish. In mammals, there are 2 known Nmu receptors. Zebrafish possess 2 orthologues of mammalian *nmur1*, designated *nmur1a* and *nmur1b*, and 1 orthologue of mammalian *nmur2*. Similar to mammalian receptor gene expression, zebrafish *nmur2* is highly enriched in widespread yet specific regions of the brain, whereas *nmur1a* is sparsely expressed in the brain (Gartlon et al., 2004). We did not observe *nmur1b* expression at stages up to 120 hpf. The effects of Nmu administration on physical activity are absent in Nmur2 knockout mice, indicating that Nmur2 is required to mediate Nmu-induced locomotor activity (Peier et al., 2009, Zeng et al., 2006). Similarly, we find that locomotor phenotypes induced by Nmu overexpression is abolished by *nmur2*$^{-/-}$ null mutation, whereas Nmu overexpression-induced hyperactivity persists in *nmur1a*$^{-/-}$ and *nmur1b*$^{-/-}$ mutant fish. Thus, the role of Nmur2 as the primary mediator of Nmu-induced locomotor activity is conserved from mammals to zebrafish.

2.3.2 Role of Nmu/Nmur2 in regulating sleep/wake architecture

Our analyses of sleep/wake architecture further refine Nmu's previously established role in regulating physical activity in mammals. We found Nmu overexpression consol-

idates waking bouts into periods of prolonged hyperactivity and shortens and reduces initiations to sleep. The effect of Nmu on sleep/wake architecture has not been extensively examined in other species, but our results are consistent with the effect of Nmu acute icv injection on sleep/wake of rats, namely prolonged wakefulness and disrupted sleep (Ahnaou & Drinkenburg, 2011).

Our study is the first to examine the role of Nmu receptors in regulating sleep/wake architecture. Although the rodent study by Ahnaou & Drinkenburg (2011) proposed that their central administration of Nmu peptide likely targeted the brain-expressed Nmur2, they were unable to rule out the possibility that these effects could be mediated by *nmur1* (which has been detected in the rodent brain, albeit at lower abundance), or that administered Nmu could target peripherally expressed receptors, or that their results might reflect endocrine signaling mechanisms downstream of Nmur2 or Nmur1. In this study, we found that neither *nmur1a*$^{-/-}$ nor *nmur1b*$^{-/-}$ fish exhibit abnormalities in sleep/wake architecture. Importantly, we found that Nmu overexpression does not disrupt sleep/wake architecture in *nmur2*$^{-/-}$ fish, but that the Nmu-induced sleep/wake phenotypes persist in *nmur1a*$^{-/-}$ and *nmur1b*$^{-/-}$ fish. Thus, we have ruled out Nmur1a and Nmur1b as required mediators of Nmu-disrupted sleep/wake architecture. We also observed for the first time that direct disruption of Nmur2 signaling in wild-type fish by Nmur2 antagonist R-PSOP (Liu et al., 2009) resulted in a dose dependent increase in sleep, as well as increases in the number of sleep transitions. These experiments indicate that the function of endogenous Nmu signaling is to promote and consolidate the wake state. Given the prevalence of insom-

nia in sleep disorders (Colten & Altevogt, 2006), Nmur2 antagonists such as R-PSOP could fulfill an unmet therapeutic need for individuals who have difficulty initiating or maintaining sleep.

Although we did not observe a locomotor phenotype in *nmur2*$^{-/-}$ mutant fish (or in *nmur1a*$^{-/-}$ or *nmur1b*$^{-/-}$ fish) the sleep/topor phenotype produced by RPSOP is consistent with the overexpression phenotype. Similar to our findings in zebrafish, no locomotor phenotypes were observed in *nmur2*$^{-/-}$ mice. We also did not observe a locomotor phenotype in the *nmu* mutant. This particular result was somewhat surprising because of the Nmu overexpression phenotype, and because mice possessing a null homozygous mutation of *nmu* exhibit more than a 50% decrease in locomotor activity (Hanada et al., 2004).

In zebrafish, the aforementioned discrepancies between the observed locomotor phenotypes in inducible models and the lack of locomotor phenotypes in developmental experiments might be explained partly by developmental changes that compensate for the constitutive loss of *nmur2*. Notably, the lack of observed behavioral phenotypes in our developmental loss of function models underscores the advantage of using an inducible system to identify hits in a phenotypic screen. A similar discrepancy exists among mammalian studies, in which pharmacological application of Nmu increased locomotor activity, whereas a transgenic mouse with constitutive nmu overexpression did not exhibit any locomotor phenotypes (Kowalski et al., 2005). Alternatively, the inducible manipulations might engage different mechanisms than chronic manipulations that could affect development.

2.3.3 A revised hypothesis of Nmu downstream signaling mechanisms

Multiple lines of evidence in mammalian systems point to a role for CRH as an essential effector of hyperactivity and anorexia of Nmu-treated rodents. Hanada et al. (2001) first predicted that Nmu's effects might be mediated by glucocorticoid signaling via the HPA axis. Additionally, a number of studies have linked the Nmu system to HPA axis signaling (reviewed by Malendowicz et al., 2012), although whether the endocrine effects are primary to Nmu administration and whether the effects seen in *in vitro* and peripheral administration studies are recapitulated by the actions of endogenous brain sources of Nmu remain unclear. The zebrafish is a useful system to study HPA axis signaling, and glucocorticoid signaling and the physiological role of glucocorticoid signaling in mediating stress is already present at early embryonic stages of zebrafish (Wilson et al., 2013). In this study, we found that Nmu overexpression phenotype functions independently of a functional glucocorticoid receptor in zebrafish. This result conflicts with the hypothesis that Nmu behaviors are manifested as a classical HPA stress response. However, it is important to point out that the mammalian and zebrafish data are not at odds because no one has tested whether GR is necessary for Nmu phenotypes in mammals. Together with data from mammalian literature, this study underlines the importance of testing other candidate mechanisms that may function downstream or parallel to Crh. The requirement of Crh for the Nmu-induced hyperactivity has not yet been tested in zebrafish, but based on the high degree of similarity of the Crh system and HPA axis between zebrafish

and mammals, we think it is reasonable to make this tentative assumption for the purposes of formulating some testable models.

Thus, assuming Crh is required for the zebrafish locomotor phenotype, there are 2 main alternative hypotheses to be tested. In one scenario, Nmu-induced sleep/wake phenotypes are mediated via alternative signaling pathways downstream of the HPA axis, such as glucocorticoid signaling at the mineralocorticoid receptor. This may be tested in the same manner as our *gr* mutant experiment. In a second scenario, Nmu's actions on locomotor behavior are mediated by HPA axis-independent, extra-hypothalamic Crh neurons that, though understudied, are well-documented. Again, this may be tested by loss-of-function manipulations to identify specific Crh populations that are required for Nmu to produce a phenotype. However, more precise techniques are needed to restrict manipulations at the neuron level as opposed to the genomic manipulations performed in this study.

So far, our data on HS-Nmu induced *cfos* expression support the second hypothesis, although it is possible that there are parallel mechanisms, and our data do not rule these out. Our key observation is that Nmu overexpression results in specific activation of brainstem Crh nuclei, as well as strong activation within the forebrain. What does this *cfos* activation patterns suggest about Nmu mechanism? It is tempting to speculate that Nmu overexpression coordinates the activation of forebrain arousal via activation of Crh neurons participating in the ascending pathways of brainstem arousal nuclei. Indeed, Crh is expressed in the locus coeruleus and parabrachial nuclei of mammals, and Crh has been shown to directly activate arousal-promoting

noradrenergic neurons of the locus coeruleus. Furthermore, central or systemic Crh administration increases wakefulness and decreases sleep in rats (Ehlers & Kupfer, 1987, Marrosu et al., 1990).

Future studies that colocalize Nmur2 with Cfos, Crh, and other neurons are required to determine which are the direct and relevant Nmu signaling pathways. Thanks to the optical transparency of the larval zebrafish, one possible approach is to non-invasively photo-ablate Crh neurons with a genetically-encoded fluorescent reporter. The requirement of brainstem Crh neurons in mediating Nmu overexpression phenotypes may be tested in this manner. A potential downside of this approach is that photo-ablation that targets Crh neurons might unintentionally extend to ablation of neighboring cell populations or passing neurites, but this can be determined empirically in future experiments. Additionally, more specific manipulations of Nmu neurons (as opposed to ubiquitous overexpression of the Nmu gene) will help to dissect the Nmu circuit.

2.3.4 Zebrafish as a minimalist model to dissect physiological functions of brain Nmu system

Although the present study provides evidence for a role of Nmu in regulating sleep/wake behavior, studies in other systems implicate Nmu in diverse physiological and pathophysiological roles, including energy homeostasis, stress, circadian rhythm, smooth muscle contraction, immunity, bone formation, gut ion transport, cardiovascular function, obesity, inflammation, and cancer (reviewed by **?**). The many physiological

functions attributed to Nmu are not surprising considering the many diverse tissues in which Nmu and receptors have been detected. In mammals it has been particularly difficult to determine the relative contributions of central or peripheral sources of Nmu to various physiological functions.

In this study, we observed CNS-restricted expression of *nmu* and receptors in the larval zebrafish. Although we cannot rule out the possibility that our *in situ* hybridization method lacked sensitivity or access to specific tissues (e.g. the spinal cord is known to be difficult to stain in whole mount developmental stages later than 72 hpf), in our hands, no peripheral expression of *nmu* or receptors was observed even in 48 hpf zebrafish, when trunk and spinal cord tissues are readily stained in whole-mount preparations. Of particular note, we observed that *nmur1a* expression is highly restricted to a single bilateral cluster; at 5 dpf we observed only 10 neurons in the hypothalamus. This is a striking result because mammalian *nmur1* is also very sparsely expressed in the brain, but it is overwhelmingly represented in peripheral tissues. Although further histological work is needed to determine the level and the developmental timing of CNS-restricted expression of Nmu and receptors in zebrafish, we tentatively suggest that larval zebrafish may serve as a useful, minimalist Nmu system to clarify the specific role of Nmu in the central nervous system independently of peripherally expressed Nmu.

2.3.5 Outlook

In summary, our findings here provide potential insights for re-interpreting mammalian data and highlight the need to identify alternative pathways through which the neuropeptide Nmu and its receptor Nmur2 act within the central nervous system to regulate sleep and arousal. The conserved yet simple zebrafish Nmu system may provide experimental advantages, and development of new tools to specifically target and manipulate Nmu neurons in the behaving fish would help to dissect the downstream signaling mechanisms. More generally, our findings here demonstrate a productive screening and follow-up approach to identifying and clarifying the roles of neuropeptides in modulating behavior.

2.4 Acknowledgments

The work in this chapter is the result of a collaboration with Jason Rihel and David Prober, who together in Alexander Schier's laboratory, designed and performed the experiments for Phase 1 of the genetic overexpression screen. Phase 2 of the screen was conducted collaboratively across the Prober and Schier laboratories. Cindy Chiu designed, performed, and analyzed all Nmu experiments. TALEN and ZFN mutants were generated in collaboration with David Prober. Heat-shock overexpression stable lines were generated in collaboration with David Prober and Viveca Sapin. Brett Niles, Alex Mack Cruz, Kenna Molinder, Axel Dominguez, and Jae Chu provided excellent technical assistance.

Chapter 3

A chemigenetic tool to manipulate neurons in behaving zebrafish

THE complexity of mammalian neural circuits and the difficulty of manipulating these circuits present major challenges in deciphering the neural mechanisms that regulate sleep and arousal. In contrast, the conserved yet relatively simple nervous system of zebrafish larvae presents an opportunity to characterize the basic neural mechanisms that regulate vertebrate sleep.

A variety of transgenic tools have recently been developed that allow the stimulation or inhibition of genetically defined neural populations. So-called optogenetic tools allow genetically specified neurons to be stimulated or inhibited by specific wavelengths of light (Mattis et al., 2012). In principle, zebrafish larvae are well suited for this technology due to their optical transparency, which is a significant advantage over non-transparent organisms such as rodents, which typically require the use of fiber optics to deliver light to specific brain regions. Indeed, this technology has been used to functionally characterize the roles of specific neurons in sensory and motor control in restrained zebrafish larvae (Arrenberg et al., 2009, Douglass et al., 2008, Wyart

et al., 2009). However, zebrafish larvae exhibit robust behavioral responses to the light stimulus, which can be problematic for experiments using freely behaving larvae (Zhu et al., 2009). Alternative approaches using transgenes that modulate neural activity in the presence of specific small molecules (Alexander et al., 2009, Arenkiel et al., 2008, Magnus et al., 2011, Szobota et al., 2007) or at specific temperatures (Pulver et al., 2009) avoid the confounding effects of light, but have yet to be tested in zebrafish.

3.1 Experimental Procedures

3.1.1 Generation of transgenic zebrafish

Tg(hcrt:TRPV1-RFPT): The rat TRPV1 channel containing the E600K mutation, which increases sensitivity to capsaicin by over 10-fold (Jordt et al., 2000), was fused to TagRFP-T (Shaner et al., 2008) and cloned downstream of the zebrafish 1 kb hcrt promoter (Faraco et al., 2006). The entire cassette was flanked by Tol2 transposase arms. Transgenic lines were generated using the Tol2 transposase method (Takayasu et al., 2006).

3.1.2 Drug treatment

Just prior to treatment, working concentrations of 1μM or 10μM Capsaicin (Csn, Sigma) were diluted into embryo medium E3 from a frozen stock of 100mM Csn in dimethyl sulfide (DMSO). All treatment conditions contained a final concentration

of 0.05% DMSO. In behavior experiments, larvae were treated with Csn just prior to lights-off at the start of the behavioral assay.

3.1.3 *in situ* hybridization and immunofluorescence

Zebrafish samples were fixed in 4% paraformaldehyde/PBS for 12-16 hours at room temperature. Fluorescent *in situ* hybridizations were performed using 2,4-dinitrophenol. (DNP)-labeled riboprobes and the TSA Plus DNP System (PerkinElmer, Wellesley, MA). Plasmid containing *cfos* (genbank clone CA787334, 870 bp) expressed sequence tag was used for riboprobe synthesis.

3.1.4 Locomotor Behavioral Assay

Larval zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5°C with lights on at 9 am and off at 11 pm. In brief, individual larvae were placed into each well of a 96-well plate (7701-1651, Whatman) containing 650 μL E3 embryo medium. Locomotor activity was monitored for 72 hours using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch Monochrome camera (LTC0385, Bosch) fitted with a fixed-angle megapixel lens (M5018-MP, Computar) and infrared filter. The movement of each larva was recorded using the quantization mode, and data from two cameras were simultaneously collected by one computer. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and illuminated with white lights from 9 am to 11 pm. 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, 29; burst, 15; freeze, 3; bin size, 60 seconds. Data were processed using custom PERL and Matlab (The Mathworks, Inc) scripts. Definitions of sleep/wake parameters are described by Prober et al. (2006).

3.2 Results

In this study, we set out to develop a chemigenetic technique that could be used to activate genetically-specified neurons and to elicit changes in the sleep/wake behavior of larval zebrafish. We chose to use the rat transient receptor potential cation channel subfamily V member 1 (TRPV1), which is a cation channel that is activated by the small molecule capsaicin (Caterina et al., 1997). Rat TRPV1 offers a potentially elegant way to non-invasively and specifically target neurons in zebrafish, because the zebrafish TRPV1 ortholog contains a mutation that renders it insensitive to capsaicin (Jordt & Julius, 2002, Gau et al., 2013). Therefore, we expected that in zebrafish, only genetically-specified neurons which express the rat TRPV1 should be activated by capsaicin treatment. Furthermore, because larval zebrafish have not yet developed a blood-brain barrier, we could easily use non-invasive bath application of capsaicin to activate TRPV1-expressing neurons without disruption to fish sleep/wake behavior.

3.2.1 Generation of a Hcrt-TRPV1 transgenic zebrafish

Our motivation for this study was to develop a tool which could be used to study the neural circuitry of sleep in zebrafish. Therefore, we targeted TRPV1 to neurons that express Hypocretin (Hcrt), a neuropeptide which was previously demonstrated to regulate sleep/wake behavior in larval and adult zebrafish (Prober et al., 2006, Elbaz et al., 2012, Yokogawa et al., 2007). We generated a transgenic line Tg(hcrt:TRPV1-RFPT), that expresses a fusion protein of rat TRPV1 and red fluorescent protein under control of the Hcrt promotor (Figure 3.1). We characterized the TRPV1 expression in our transgenic line by colocalizing RFPT expression with the previously established Hcrt neuron fluorescent reporter line, Tg(hcrt:GFP) (Prober et al., 2006). We detected TRPV1-RFPT in 85% of GFP-positive Hcrt neurons. Conversely, we detected GFP in 95% of TRPV1-RFPT-positive neurons. Thus, our Tg(hcrt:TRPV1-RFPT) zebrafish line shows nearly comprehensive and highly specific expression of the transgene in Hcrt neurons.

3.2.2 TRPV1-dependent activation of Hcrt neurons

After confirming the specific expression of TRPV1 in our transgenic line, we next asked whether we could activate Hcrt neurons with TRPV1. Using *cfos* expression as a readout of neuronal activation, we found that 10μM and 1μM doses of capsaicin could activate over 95% of Hcrt neurons (Figure 3.2). In our wild-type negative control conditions, we detected little to no *cfos* activation in Hcrt neurons after treatment with capsaicin. As an additional negative control, we showed that when Hcrt neurons

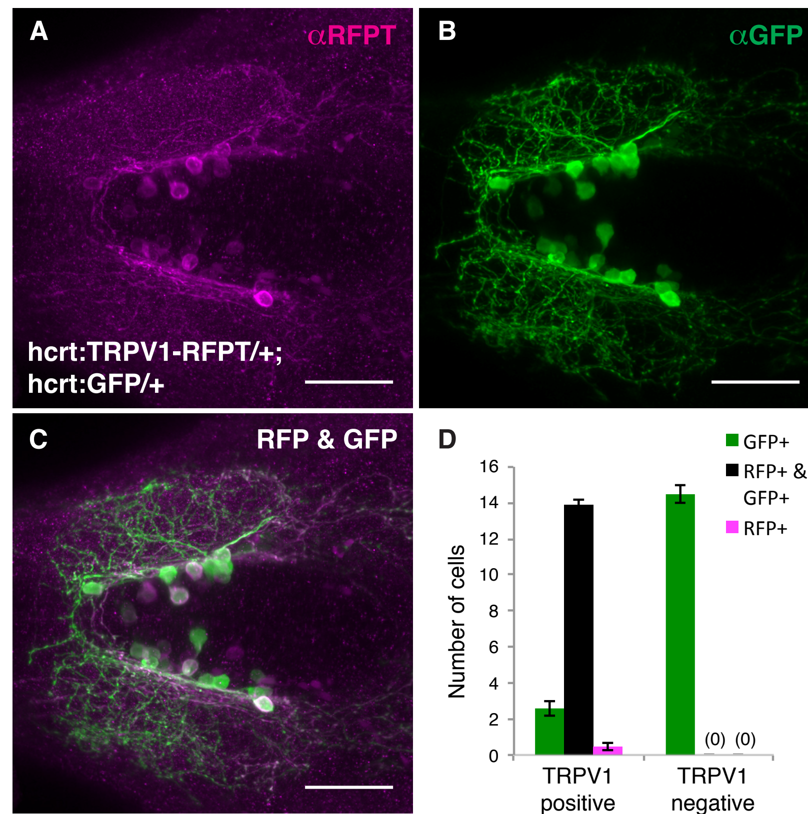

Figure 3.1: Expression of TRPV1-RFPT in zebrafish Hcrt neurons. A-C. A representative sample of Tg(hcrt:TRPV1-RFPT) expression. Images show a maximum intensity projection of a 50μm thick confocal z-stack. Pseudo-colored images show the confocal red (TRPV1-RPFT) channel (A), green (GFP) channel (B), and both channels merged (C). D. RFPT-positive and GFP-positive cell counts in double transgenic Tg(hcrt:TRPV1-RFPT) and Tg(hcrt:GFP) fish and a comparison to control single transgenic Tg(hcrt:GFP) fish. Scale bar =20μm.

are activated with a 10μM dose of capsacin in the Tg(hcrt:TRPV1-RFPT) line, a neighboring population of neurons (Qrfp) that does not express Hcrt or TRPV1 shows little to no activation. Thus, we show that it is possible to specifically activate Hcrt neurons in the newly established transgenic line.

3.2.3 Dose-dependent ablation of Hcrt neurons

In the aforementioned *cfos* activation experiment, we noticed a hint of abnormal morphology in the Hcrt neurons that were activated by a 10μM capsaicin dose (Figure 3.2). Specifically, TRPV1-positive, 10μM capsaicin-activated Hcrt neurons exhibited slightly bloated somas and blebby neurites, which are indicative of deteriorating cellular integrity. We reasoned that this might be a specific result of hcrt:TRPV1-dependent excitation at 10μM capsacin because we did not observe this abnormal morphology in Hcrt neurons treated with 1μM capsaicin, or in Qrfp neurons treated with 10μM capsaicin. We wondered whether a more prolonged activation using TRPV1 plus 10μM Capsaicin might be a useful tool to ablate neurons, so we performed a 24 hour time course of Hcrt cell number to test this.

We found that indeed, there was a dose-dependent ablation of Hcrt neurons (Figure 3.3). At 10μM capsaicin dose, 50% of Hcrt-GFP neurons were absent within 1 hour of capsaicin treatment. After 24 hours, 60% of Hcrt-GFP neurons were absent. We suspect this is an underestimate of the number of ablated cells due to persistence of GFP because an assay of Hcrt neurons by immunohistochemical detection of endogenous protein showed 80% of cells were absent after the same treatment of

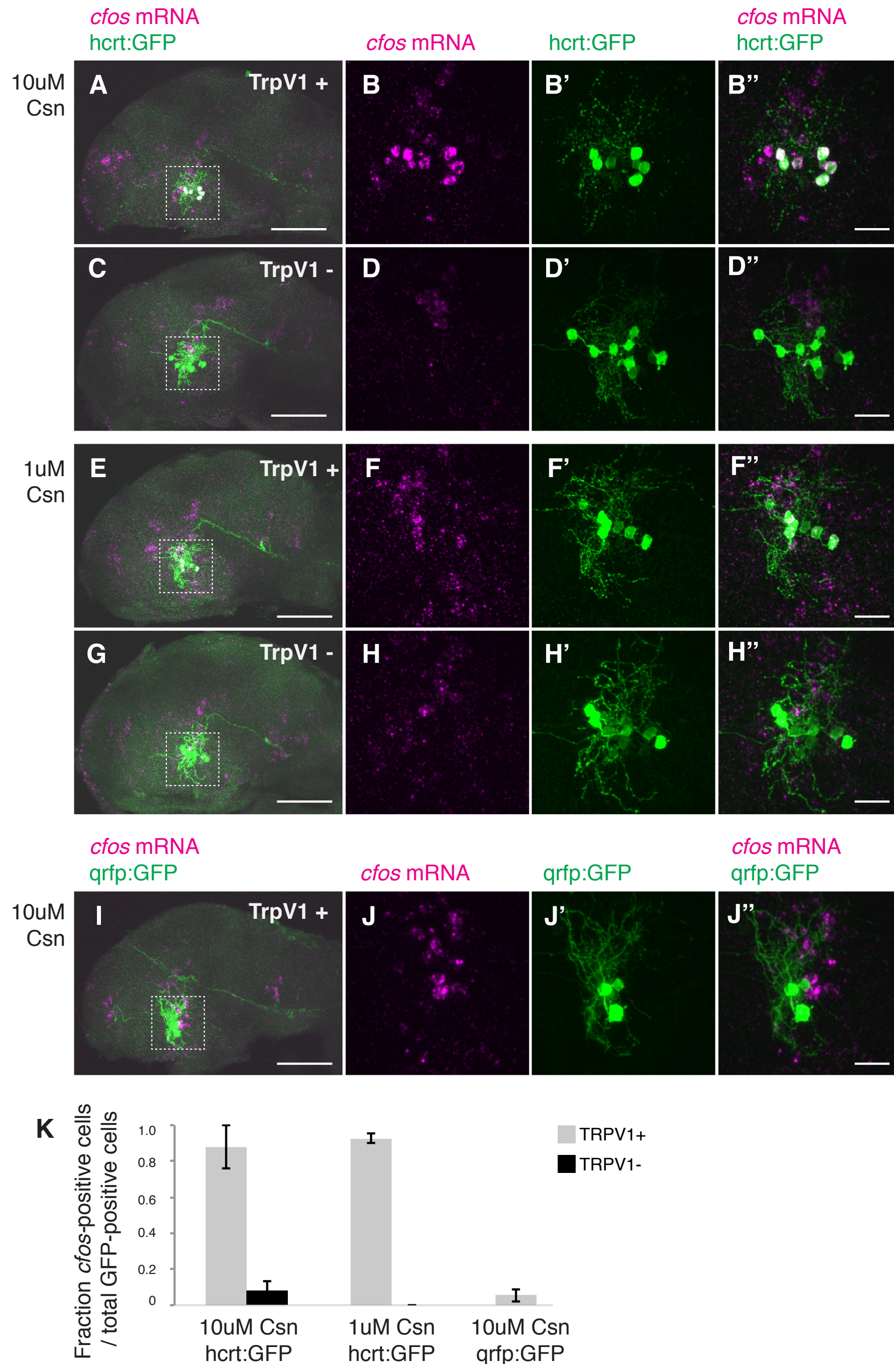

Figure 3.2: Specific activation of Hcrt neurons in the TRPV1 transgenic. A-J. Representative images of Hcrt (A-H) or Qrfp (I-J) neuron morphology, detected by anti-GFP IHC (pseudo colored green), and *cfos* expression, detected by fluorescent ISH (pseudo colored magenta), in TRPV1-positive (TRPV1+, A-B, E-F, I-J) or -negative (TRPV1-, C-D, G-H) larval zebrafish brains after a 20-minute dose of 10μM (A-D, I-J) or 1μM (E-H) capsaicin. Whole brain (A,C,E,G,I, Scale=100μm) and higher magnification views of the boxed areas (B,D,F,H,J, Scale=20μm) are oriented rostral=left and caudal=right. Images are maximum intensity projections of 40μm total thickness confocal z-stacks. K. Summary quantification of average fraction of *cfos*-positive cells from the conditions shown in A-J.

10μM capsaicin for 24 hours. This effect was not due to the capsaicin itself, because the 10μM capsaicin dose did not affect the Hcrt cell numbers in TRPV1-negative fish. Importantly, 1μM capsaicin did not reduce Hcrt cell numbers, even though we observed activation of neurons at 1μM capsaicin in the previous experiment. Therefore, we serendipitously ended up with a single tool with two applications: neuronal activation at 1μM capsaicin, and neuronal ablation at 10μM capsaicin.

3.2.4 TRPV1 activation of Hcrt neurons produces behavioral arousal and insomnia

Next, we tested whether activation and ablation of Hcrt neurons with TRPV1 results in behavioral phenotypes that are predicted by respective GOF and LOF models of Hypocretin function. Because the effects of genetic activation and ablation of the Hcrt neurons have been characterized in larval zebrafish, we used the behavioral readouts of previous studies to test the functionality of our TRPV1 transgenic.

Previously, Prober et al. (2006) used a Hcrt genetic overexpression model to establish that the zebrafish Hcrt system promotes wakefulness and arousal in larval zebrafish. Thus, we hypothesized that activation of Hcrt neurons with TRPV1 would result in phenotypes similar to those observed in the Hcrt overexpression model. Indeed, we found that activation of Hcrt neurons using 1μM capsacin in the hcrt:TRPV1-RFPT transgenic resulted in significantly increased locomotor activity, decreased sleep, hyperactivity, and lower propensity to initiate a sleep bout (Figure 3.4).

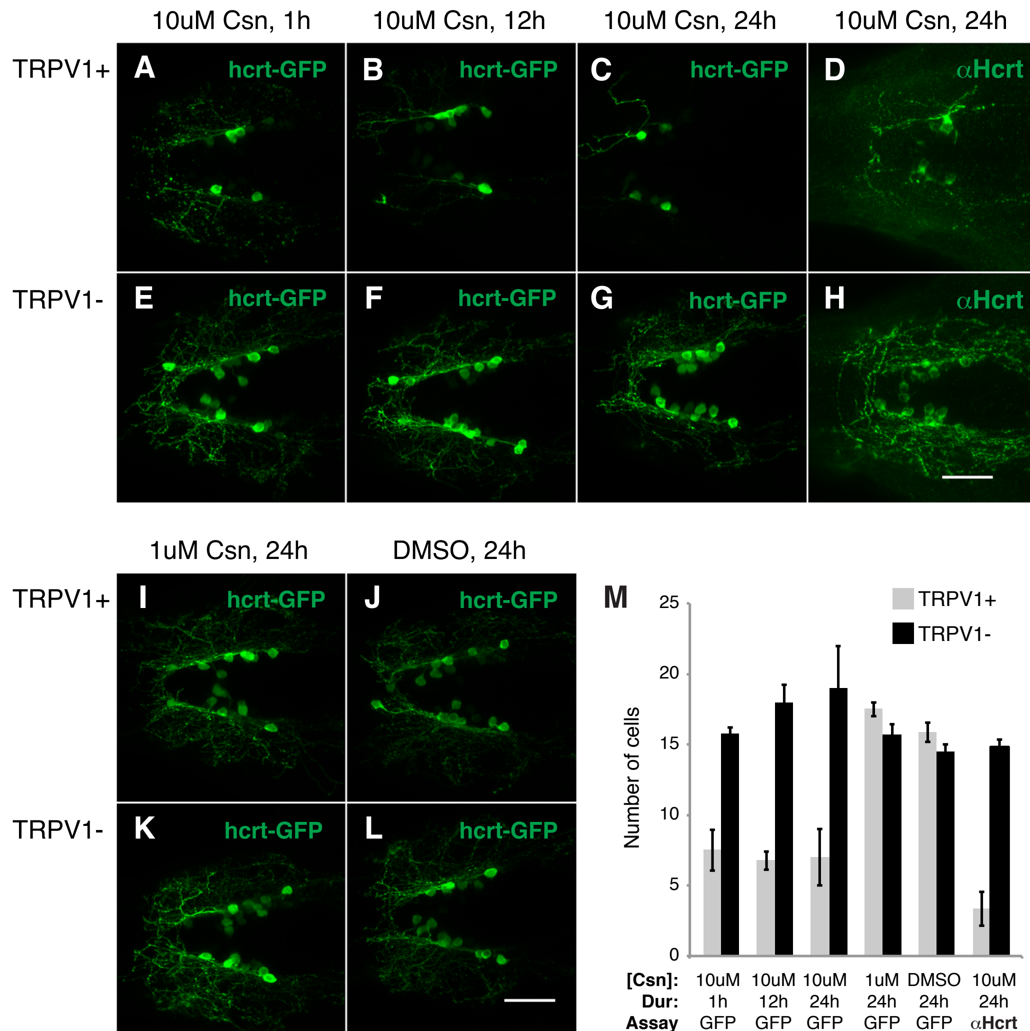

Figure 3.3: Dose-dependent ablation of Hcrt neurons in the TRPV1 transgenic. A-J. Representative images of Hcrt neuron morphology, detected by anti-GFP IHC (A-C,E-G,I-L) in hcrt:GFP; hcrt:TRPV1-RFPT double transgenic (A-C, I-J) or hcrt:GFP single transgenic (E-G,K-L) zebrafish. For an independent measure of Hcrt neurons, endogenous Hcrt protein was detected by IHC in transgenic Hcrt-TRPV1-RFPT (D) or WT (H) zebrafish. Samples are oriented rostral=left and caudal=right. Images are maximum intensity projections of 40μm thick confocal z-stacks. M. Summary quantification of average cells detected in the conditions shown in A-L. Scale=20μm).

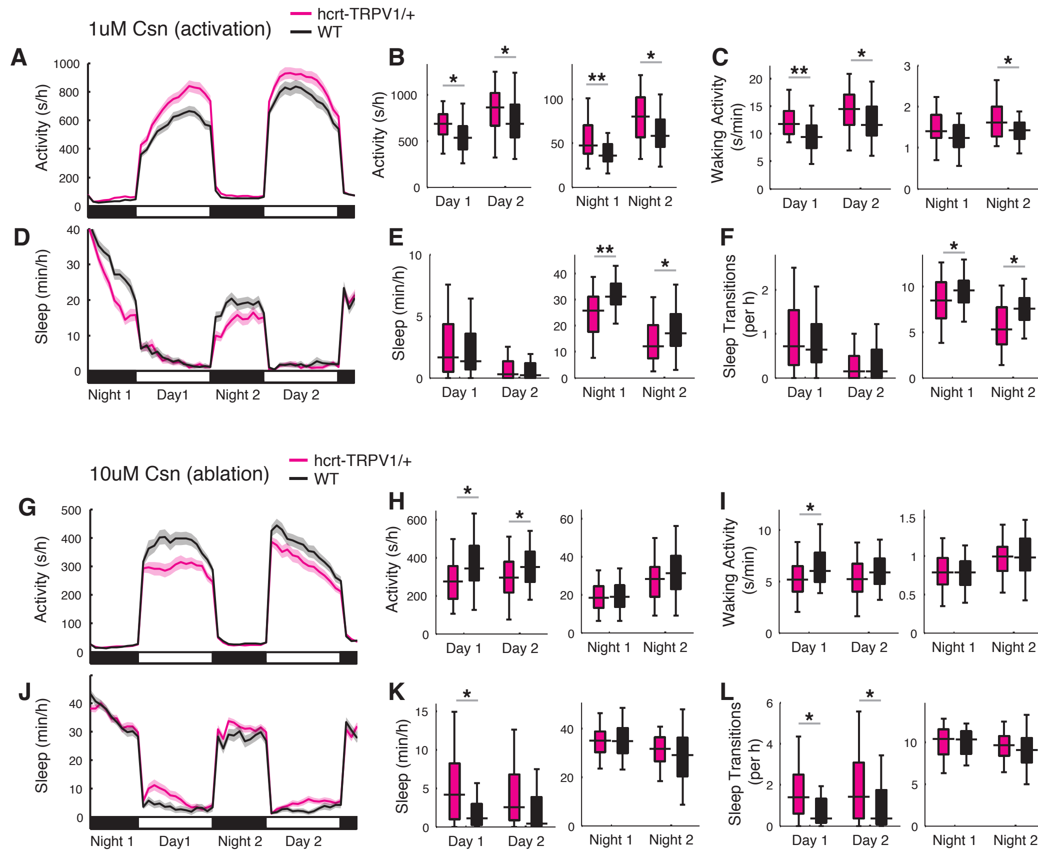

Figure 3.4: TRPV1 activation and ablation of Hcrt neurons in behaving zebrafish. A-F. Behavioral phenotypes following activation of Hcrt neurons with 1μM Csn in hcrt-TRPV1-RFPT (magenta) zebrafish and comparison phenotypes of WT siblings identically treated and simultaneously recorded. G-L. Behavioral phenotypes following ablation of Hcrt neurons with 1μM Csn in hcrt-TRPV1-RFPT (magenta) zebrafish and comparison phenotypes of WT siblings (black) that were identically treated and simultaneously recorded. Scale=50μm.

3.2.5 TRPV1 ablation of Hcrt neurons produces torpor and narcolepsy-like behavior

A genetic model of Hcrt ablation in larval zebrafish predicts that ablation of Hcrt neurons by 10μM capsaicin should reduce activity and increase sleep transition number, i.e. fragment sleep/wake (Elbaz et al., 2012). Indeed, we observed significant decreases in locomotor activity, increased sleep, and more frequent day time initiations of sleep following 10μM capsaicin/TRPV1-mediated ablation of Hcrt neurons (Figure 3.4). Strikingly, the zebrafish neuron ablation phenotype resembles narcolepsy in humans, which is a pathological condition associated with pronounced reductions in Hcrt cell number.

3.3 Discussion

In this study, we validate the use of TRPV1 channels for specific activation and ablation of neurons in behaving zebrafish. This technology offers some important advantages over optogenetics, namely that this chemigenetic technique can be used without disruption to sleep behavior or other light-dependent behaviors. We also anticipate that this technology will play a complementary role with existing genetic methods that report neuronal activity by release of light. For example, aequorin, a luminescent Ca2+ indicator, has recently been developed to report the activity of neurons in behaving zebrafish (Naumann et al., 2010). Additionally, TRPV1 can be used in combination with fluorescent Ca2+ indicators such as GCaMP, whose use in

combination with optogenetic tools such as ChR2 is complicated by the ability of blue light to both activate ChR2 and excite GCaMP fluorescence. It will be interesting to use our newly established hcrt:TRPV1-RFPT transgenic in conjunction with such reporters of neural activity to understand how Hcrt neurons affect other neural circuits and behavior. Likewise, it will be interesting to study how Hcrt neuron signaling during behavior is altered by the activation of pre-synaptic neuron populations using TRPV1. More generally, the chemigenetic technology developed in this proof-of-principle study provides a springboard for future studies to characterize neural circuits and the behaviors that they regulate.

3.4 Acknowledgments

Cindy Chiu designed, performed, and analyzed all experiments with assistance from Caltech undergraduate Sohini Khan. The Tg(hcrt:TRPV1-RFPT) line was generated in collaboration with David Prober. Viveca Sapin, Alex Mack Cruz, Kenna Molinder, Axel Dominguez, Jae Chu, and Brett Niles provided excellent technical assistance.

Chapter 4

Conclusion

WHY and how we sleep is an enduring mystery. To understand the function and regulation of sleep, we need to identify the key players and develop and employ methodologies to interrogate the relevant brain systems. This thesis represents two of the studies that I conducted in graduate school to tackle these broad issues. In these concluding remarks, I will briefly summarize and discuss the significance of my results, and I will propose a few directions for future research.

4.1 Significance of results

4.1.1 Discovery of a new sleep/arousal pathway

In Chapter 2, I described the discovery and characterization of a new regulatory mechanism of sleep, the Neuromedin U/ Nmur2 pathway. In characterizing this pathway and downstream components in zebrafish, we found evidence that argues against the previously proposed hypothesis that Nmu signaling with respect to behavior is manifested as a stress response that is mediated by the hypothalamic-pituitary-adrenal

axis. Instead we found new evidence that Nmu signaling may be mediated by arousal centers in the brainstem. Thus, the main contribution of this work is that it identifies a sleep-regulating circuit and generates new testable predictions about how sleep is regulated by this circuit. Secondarily, we found that the larval zebrafish represents a conserved but much simpler model of the vertebrate Nmu system, because in zebrafish, Nmu and its receptors do not appear to be represented in the periphery as in mammals. This observation therefore identifies the zebrafish as a useful comparative model to clarify the relative contributions of central and peripheral Nmu in other aspects of animal physiology in which it has been implicated.

4.1.2 Development of a new technology to functionally dissect sleep/arousal pathways in zebrafish

The study described in Chapter 2 highlighted a need for more precise methodologies to manipulate neural circuitry underlying sleep. However, the currently available tools in zebrafish preclude the study of sleep behavior because they are either invasive (e.g. electrophysiology) or require visible light which disrupts sleep behavior (e.g. optogenetics). Thus, in a study described in Chapter 3, we addressed this need by developing a chemigenetic method. Our method employs a cation channel that is activated by a behaviorally non-disruptive chemical to manipulate neurons that are specified by genetics. We validated this technique and performed proof-of-principle assays to show that this technology can be used in the freely behaving fish. Although our motivation for developing this technology was to enable future studies of sleep

neural circuitry in zebrafish, our method has broad applications in any zebrafish neurobehavioral studies, with a particular advantage for the study of light-dependent behaviors, such as feeding (zebrafish primarily use vision to hunt for food).

4.2 Future directions

The work here generates numerous avenues of future research, and I will describe just a few of the possibilities here.

4.2.1 Identifying functionally connected sleep/wake circuits

Our study in Chapter 2 opened up a clear line of research questions regarding the neuronal and genetic mechanisms that are downstream of the Nmu/Nmur2 sleep regulation pathway. One important question that we hope to address with the newly developed zebrafish TRPV1 technology is which neurons are functionally connected to the Nmu/Nmur2 sleep regulatory circuit. A starting point would be to reproduce the HS-Nmu *cfos* results using the TRPV1 method. The steps would be to 1) identify an Nmu gene enhancer region that can drive expression in endogenous Nmu neurons, 2) create a Tg(nmu:TRPV1) that expresses TRPV1 in Nmu neurons, and 3) determine which neurons are activated by Nmu neuron activation. Our hypothesis is that activation of Neuromedin U neurons will phenocopy the ubiquitous overexpression of Neuromedin U. Thus, we expect to see that in addition to Nmu neurons themselves, brainstem Crh neurons and neurons in the forebrain are activated. Any differences in *cfos* expression patterns that are observed following HS-Nmu versus nmu:TRPV1

induced activation might be interpreted as off-target (in the case of HS-Nmu specific signals) versus endogenous circuit-related, since the nmu:TRPV1 experiment should be more restricted in its activation. Also, colocalization of *nmur2* to any *cfos* positive neurons will help to identify which neurons are likely directly or indirectly activated by Nmu neurons or Nmu overexpression.

If this initial step is confirmed, it will be interesting to use additional types of neural activity reporters other than *cfos*, because *cfos* expression is transient and does not give a comprehensive picture of the identities of neurons that respond with a longer or delayed time course of activation following a stimulus such as TRPV1. Also, in some types of neurons, cfos is thought to be completely uncorrelated or anti-correlated with neuron depolarization. Generally speaking, the best way to directly record systems-level neural activity is to use electrophysiology, but this has not been successful in the larval zebrafish for reasons of scale incompatibility between larval zebrafish brains and current electrode technology. As an alternative, a promising way to search brain-wide for neurons that are functionally connected to Nmu neurons is a recently developed *in vivo* zebrafish whole-brain functional imaging approach. This technology delivers single-cell resolution of zebrafish whole-brain activity using genetically-encoded calcium indicators. By combining these with our TRPV1 technology, we could potentially capture brain-wide responses to nmu:TRPV1 activation in order to identify which groups of neurons are activated by Nmu neurons over behaviorally relevant time scales.

Many more iterations of the reductionist approaches described in Chapter 2 and in

this section will eventually define the essential players in the Nmu/Nmur2 pathway. We anticipate that most of the genes in the zebrafish genome will be mutated in the next several years, and it will be straightforward to test whether any additional candidate genes, such as Crh, are required for Nmu overexpression to alter sleep behavior. Furthermore, as new zebrafish enhancers are identified, it will be possible to test more specific hypotheses about which particular populations of neurons, such as which Crh neurons, contribute to Nmu-mediated sleep and arousal behavior.

4.2.2 Identifying mechanisms of diurnal rhythms in zebrafish

As a diurnal vertebrate with excellent gene-editing resources, the zebrafish serves as a useful comparative system to the genetics-friendly, nocturnal mouse and the non-genetics-friendly, diurnal human. A research question that follows from this thesis is how is the Nmu sleep/arousal regulatory pathway itself regulated with regards to circadian activity patterns. An intriguing clue is that *nmu* transcription has been observed to be negatively regulated by melatonin, a neuromodulator which itself is negatively regulated by light. Thus, a simple model of how diurnal rhythms might be mediated by melatonin regulation of Nmu signaling in the zebrafish might be that during night time, high levels of melatonin inhibit Nmu production, which in turn suppresses the behavioral effects of Nmu signaling in promoting arousal. In the day time, negative regulation of Nmu is turned off, and thus the Nmu signaling pathway can actively promote arousal. Indeed, preliminary data show that in zebrafish, *nmu* expression is stronger during the day than in the night. Thus, it will be interesting

to test this hypothesis in the diurnal zebrafish using its vast armory of genetic and molecular tools. Indeed, the contribution of this pathway to regulating circadian behavior may be underestimated in mice because commonly used laboratory mouse strains produce little or no melatonin.

4.2.3 Next generation screen designs

Our genetic screen was successful in identifying an exciting avenue of sleep circuit research, but the hunt for additional sleep regulators continues. Next generation screen designs could extend the present screen of 1000 human secreted proteins by screening through unannotated regions of the zebrafish genome. This approach might be better suited for identifying any as yet unidentified secreted proteins that regulate sleep, and testing of zebrafish rather than human genes may yield more robust phenotypes in zebrafish. Another potential screen design is to use the TRPV1 activation/ablation technology developed here in combination with a bipartite driver/effector system, such as GAL4/UAS. The idea would be to screen through any GAL4 driver lines to identify enhancer-defined cells that produce an abnormal behavior when activated or ablated in the presence of capsaicin and a UAS:TRPV1 effector. This approach offers at least three important advances over our present heat-shock overexpression screen: 1) The use of GAL4 drivers should be useful for identifying sleep-regulating neural circuits that are not defined by genes encoding secreted proteins. However, the interpretation of the results might be limited by the specificity of expression of any given GAL4 driver; 2) The use of a UAS:TRPV1/capsaicin inducible system is

more behaviorally inert compared to heat shock overexpression, and this may be useful to study more nuanced aspects of sleep and arousal (e.g. free running circadian rhythms) that might be otherwise disturbed by a 1 hour heat shock treatment; 3) The UAS:TRPV1 transgene potentially offers both a gain-of-function and loss-of-function effector using the same zebrafish transgenic line. This 2-in-1 deal is advantageous both for practical reasons (cost/labor/efficiency benefits) and for scientific reasons (robust results are more easily obtained when experiments are well-controlled). All in all, we expect that TRPV1 will serve as an excellent next step for genetic screens for sleep regulators. Also, although I think sleep is a particularly fascinating topic to study, the approaches described here should be easily applied to other behavioral or non-behavioral phenotypic assays in zebrafish.

4.2.4 Differentiating different forms of sleep or arousal states and their neural mechanisms

A common theme among neuromodulatory systems is that they play multiple overlapping roles in regulating animal physiology. For example, in mammals and now in zebrafish, Neuromedin U and Hypocretin are both known to promote arousal. Additionally, in mammals, it is also known that these two arousal neuropeptides play opposite roles in feeding: Neuromedin U is anorexigenic, whereas Hypocretin is orexigenic. These differences are a major clue that Neuromedin U and Hypocretin systems engage different mechanisms to regulate behavior, and that they may also mediate distinct forms of sleep/arousal. In the future, it will be interesting to ask

if, how, and why there are different sleep/arousal states by studying the overlapping and unique functional and mechanistic aspects of neuromodulatory arousal systems such as Hypocretin and Nmu.

A straightforward extension of the approaches used in this thesis would be to study how heat-shock overexpression or TrpV1 activation/ablation of the Hypocretin and Neuromedin U systems cause similar or disimilar responses in a battery of different behavioral tests. However, the spectrum of established behavioral assays is somewhat limited in zebrafish relative to rodent systems, so development of a larger repertoire of quantitative behavioral assays in zebrafish will help with this endeavor. A feeding assay and various assays of arousal would be a good start. Additionally, it will be interesting to determine whether there are different degrees of sleep quality or different sleep states by examining whether there are any particular behavioral or physiological indicators of sleep, as has been observed in humans (e.g. atonia, or characteristic movements and brain coherence in sleep, and changes in cardiovascular function in arousal). As various zebrafish arousal and sleep states are quantitatively determined, all of the tricks of the zebrafish system can be brought to bear on understanding the mechanisms that define and control these states.

4.2.5 Deciphering endogenous and systems-level functions of Nmu and Hcrt

In my humble estimation, a major disadvantage of the aforementioned reductionist approach is that it is difficult to interpret to what extent the activation or abla-

tion manipulations reflect the kinds of signaling that occur endogenously and during normal sleep/wake behaviors (although they provide an extremely productive approach for identifying neuronal connectivity and may prove useful for modeling certain pathophysiological states such as narcolepsy in zebrafish). Therefore, a useful complementary approach would be to study the signaling of these neurons during natural behavior. This might be achieved using GFP-Aequorin reporter (Naumann et al., 2010). This technique is non-invasive, does not require excitation light, and allows an animal to freely behave while the activities of genetically specified neurons are monitored.

Additionally, it has not escaped my attention that the overexpression of Nmu activates a large population of neurons in the zebrafish forebrain, as measured by *cfos*, and my preliminary data not shown here suggest that, among other areas, there is *cfos* across the olfactory bulb following Hcrt overexpression. If the specific activation of these respective populations by Nmu and Hcrt neurons are confirmed by more specific and direct neuronal methods as described above, then it would be of great interest to examine the systems-level functions that these neuromodulators play in aspects of neural coding. For example, it would be interesting to see if Nmu and Hcrt neuromodulation participates in gain control of forebrain or olfactory neurons in order to modulate the sensitivity of zebrafish to incoming internal or sensory signals during sleep.

4.3 Concluding remarks

These are still early days for using zebrafish as a model to study sleep and arousal states, but it is already clear that zebrafish can provide new and important insights into genetic and neural mechanisms that regulate behavior. In this thesis, I employed a new high-throughput screen design to reveal new mechanisms that regulate sleep and arousal. I also developed a new tool that exploits advantageous features of zebrafish to enable new insights into the neural regulation of sleep and arousal. I expect that these developments will lead to the discovery of new mechanisms that regulate sleep and arousal states that would be difficult to obtain using other vertebrate model organisms.

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