LIGHT DEPENDENT REGULATION OF SLEEP/WAKE STATES BY PROKINETICIN 2 IN LARVAL ZEBRAFISH

Thesis by

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For my family

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ABSTRACT

Sleep is an evolutionarily conserved behavior and essential to survival. The classic two process model of sleep regulation proposes that sleep results from the interaction between circadian and homeostatic processes, but the details remain elusive. Most sleep research is performed using nocturnal rodents, and diurnal vertebrates are under-represented. It is unclear whether circadian regulatory mechanisms of sleep in nocturnal animals can be directly translated into diurnal animals. In this thesis, I first briefly describe sleep behavior and the two process model of sleep regulation, focusing on the circadian process, and then discuss the advantages of using larval zebrafish as a model to study sleep behavior in diurnal vertebrates. In Chapter 2, I characterize the role of Prokineticin 2, a proposed circadian output factor in nocturnal animals, in sleep/wake regulation in larval zebrafish. I show that, similar to nocturnal rodents, Prok2 is both necessary for daytime sleep/wake behavior and sufficient to modulate sleep/wake states in a light dependent manner. However, unlike nocturnal rodents and similar to humans, Prok2 is not required for maintaining circadian rhythmicity in larval zebrafish after removing external light cue. This result demonstrates the potential functional difference of circadian output factors in different chronotypes, and establishes larval zebrafish as an alternative model for studying circadian regulation of sleep and possibly other behaviors in humans. In Chapter 3, I describe the adaptation and development of TRP channels to manipulate neuronal activity in larval zebrafish, in an effort to expand the existing repertoire of genetic tools for studying behavior in zebrafish. I show that three TRP channels, TRPV1, TRPM8 and TRPA1, can inducibly activate specific populations of neurons in larval zebrafish by using their appropriate agonists. At high agonist

concentrations, TRPV1, can rapidly induce cell ablation. Adaptation of TRP channels for use in larval zebrafish expands the variety of behavioral experiments and combinatorial manipulation of neuronal activity that can be performed in zebrafish. In summary, this work deepens our understanding of sleep regulation, establishes larval zebrafish as an appropriate model for studying circadian regulation of sleep in diurnal vertebrates, and presents novel genetic tools for studying behavior in larval zebrafish.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

INTRODUCTION

1.1 Sleep

Sleep is a conserved behavior observed across the animal kingdom (Zimmerman et al., 2008) and is essential for survival (Cirelli and Tononi, 2008). Sleep-like states can be defined by a set of behavior criteria (Campbell and Tobler, 1984; Siegel, 2008):

- A prolonged period of quiescence occurring in specific periods of the circadian cycle – in diurnal animals (i.e., humans), sleep usually occurs at night.
- Elevated sensory arousal threshold – sleeping animals exhibit reduced responsiveness to sensory stimuli, which distinguishes sleep from quiet wakefulness. This sleep state can be reversed by strong stimuli.
- Homeostatic regulation – sleep deprived animals exhibit a need for compensatory sleep.

The function of sleep has been postulated from energy conservation and physiological restoration to memory consolidation (Mignot, 2008). Over 10% Americans suffer from chronic sleep disorders (NHLBI, 2003). However, the genetic and neural regulation of sleep behavior still remains largely a mystery (Saper et al., 2005c; Cirelli, 2009).

1.2 The two process model of sleep regulation

In 1982, Alexander Borbély proposed the "two process model of sleep regulation" that has been widely accepted as a conceptual framework of sleep regulation (Borbély, 1982). According to the model, the architecture of the sleep/wake cycle is determined by the interaction between a homeostatic process and a circadian process. The homeostatic process responds to the internal need for sleep, and the circadian process is controlled by

a circadian oscillator that responds to environment cues (i.e. light). In diurnal animals, sleep occurs during the night when the circadian drive for waking is low and the internal propensity to sleep is high.

1.3 Homeostatic process of sleep regulation

The homeostatic process regulates the internal propensity for sleep, and it increases exponentially with the duration of wakefulness and dissipates during sleep (Borbély, 1982). The homeostatic regulation of sleep functions independently from the circadian regulation because animals with lesions in the suprachiasmatic nucleus (SCN), the master circadian clock (see 1.4), continue to sleep and exhibit recovery sleep after sleep deprivation (Mistlberger et al., 1983; Tobler et al., 1983).

Several molecules have been proposed to mediate the homeostatic regulation of sleep. Adenosine, the by-product of the breakdown of adenosine triphosphate (ATP), has been identified as a mediator of sleep (reviewed in Bjorness and Greene, 2009). Several studies have shown that the adenosine level in the basal forebrain increases with prolonged wakefulness (Porkka-Heiskanen et al., 1997, 2000; Murillo-Rodriguez et al., 2004). Furthermore, adenosine agonists inhibit wake active neurons in the basal forebrain (Alam et al., 1999; Thakkar et al., 2003) and hypocretin (Hcrt) neurons in the lateral hypothalamus (Liu and Gao, 2007), and activate sleep active neurons in the ventrolateral preoptic area (VLPO) (Scammell et al., 2001; Chamberlin et al., 2003; Morairty et al., 2004; Gallopin et al., 2005; Methippara et al., 2005). Caffeine, a potent stimulant for wakefulness, functions as an antagonist of adenosine receptor and exerts its wake promoting effects through adenosine A2A receptor (Huang et al., 2005). Nitric oxide

(NO) has been shown to stimulate release of extracellular adenosine (Rosenberg et al., 2000). NO level increases in the basal forebrain upon prolonged wakefulness (Kalinchuk et al., 2010) and its production is both required and sufficient to produce sleep (Kalinchuk et al., 2006a, 2006b). Pro-inflammatory cytokines such as interleukin-1b and tumor necrosis factor-a level correlate with sleep propensity (Bredow et al., 1997; Floyd and Krueger, 1997; Taishi et al., 1998). They can increase non-REM sleep, and inactivating them reduces sleep (reviewed in Opp, 2005). Prostaglandin D2, a lipid signaling molecule, increases with increasing sleep propensity (Ram et al., 1997). Infusion of prostaglandin induces sleep (Scammell et al., 1998) possibly via adenosine A2A receptor (Satoh et al., 1996, reviewed in Huang et al., 2007). GnRH has been shown to enhance sleep in both humans (Kerkhofs et al., 1993) and other mammals (Ehlers et al., 1986; Obal et al., 1988).

In addition to the molecules that modulate sleep/wake states of animals, many efforts have been put into identifying a neuroanatomical basis of homeostatic regulation of sleep (reviewed in Saper et al., 2001). The mammalian sleep and wake promoting centers and homologous zebrafish regions are highlighted in Figure 1.1. Sleep promoting neurons have been identified in the VLPO (Sherin et al., 1996) and the median preoptic nucleus (MnPN) in the preoptic area (Gong et al., 2004). These neurons are primarily active during sleep and send inhibitory neurotransmitters GABA and galanin to wake promoting neurons (Sherin et al., 1998; Uschakov et al., 2007). The melanin-concentrating hormone (MCH) neurons in the lateral hypothalamus are active during REM sleep and activation of these neurons increases sleep in mice (Verret et al., 2003; Konadhode et al., 2013). In addition, a group of widespread GABAergic interneurons in

the cortex expressing nitric oxide synthase are active during slow wave sleep (Gerashchenko et al., 2008; Pasumarthi et al., 2010).

The arousal system consists of the Hcrt neurons in the lateral hypothalamus (Peyron et al., 2000; Mileykovskiy et al., 2005), the noradrenergic locus coeruleus in the pontine brainstem (Aston-Jones and Bloom, 1981), the dopaminergic neurons in the ventral periaqueductal grey matter (Lu et al., 2006), the serotoninergic dorsal raphe nuclei in the medial brainstem (McGinty and Harper, 1976), the histaminergic tuberomammillary nucleus in the posterior hypothalamus (Parmentier et al., 2002), and the cholinergic pedunculopontine and laterodorsal tegmental nuclei in the midbrain (Hallanger et al., 1987; McCormick, 1989) and in the basal forebrain (Lee et al., 2005). These wake promoting centers inhibit sleep active neurons and send ascending projections throughout the cerebral cortex and thalamus to abolish the synchronized low-frequency rhythms in the thalamocortical system associated with sleep and promote high frequency oscillation (Steriade et al., 1993). The mutual inhibition between the wake and sleep promoting neurons results in distinct sleep/wake states in animals (Saper et al., 2005c).

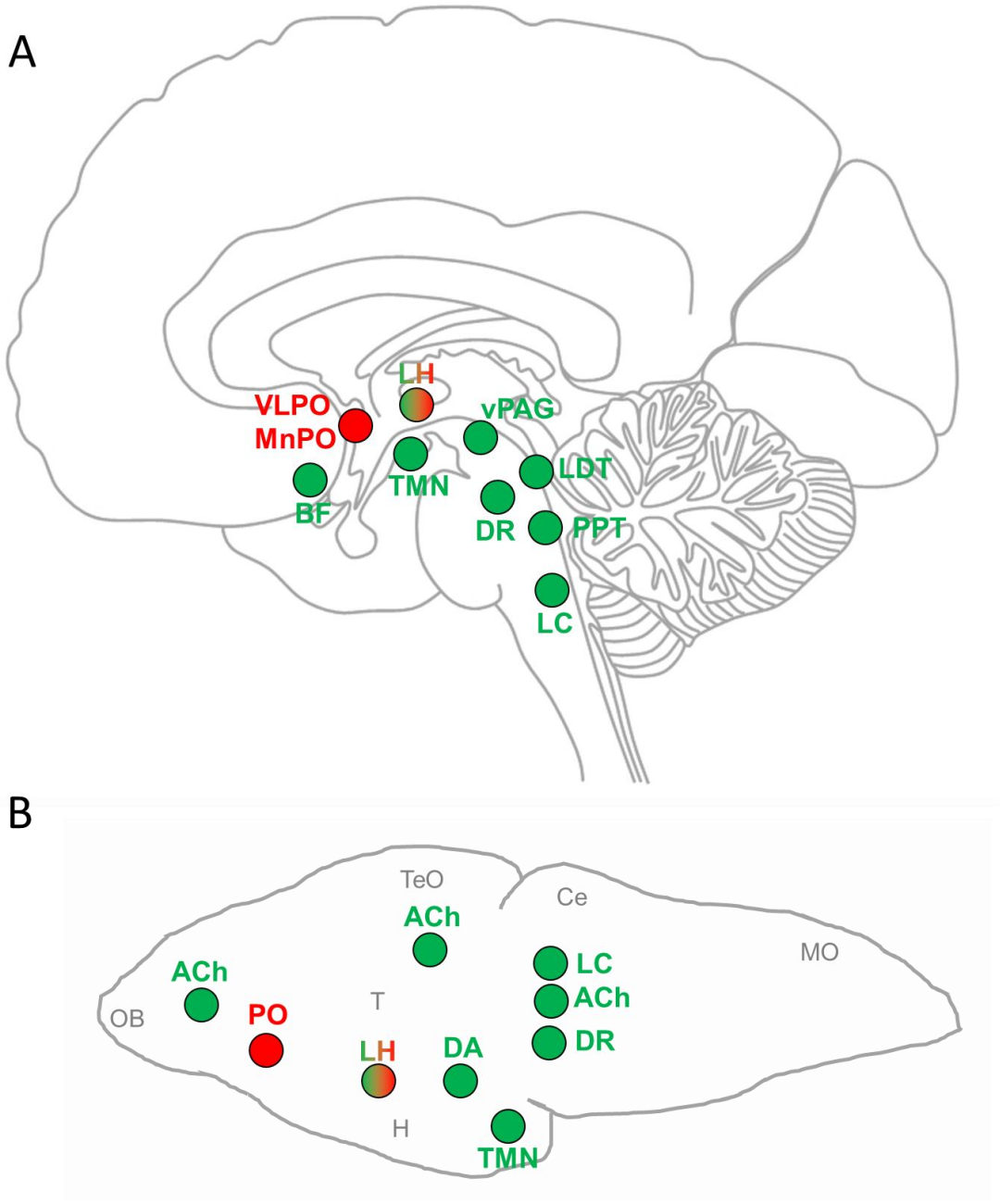


Figure 1.1. Neural populations that regulate sleep and wakefulness. A, sagittal view of a human brain. B, homologous structures in a sagittal view of larval zebrafish brain. Green circles indicated wake-promoting nuclei and red circles indicate sleep-promoting nuclei. Abbreviations: VLPO, ventrolateral preoptic nucleus; MnPO, median preoptic nucleus; LH, lateral hypothalamus; vPAG, ventral periaqueductal grey; TMN, tuberomammillary nucleus; BF, basal forebrain; DR, dorsal raphe nuclei; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine nucleus; PO, preoptic nucleus; DA, dopaminergic neurons; Ach, cholinergic neurons; OB, olfactory bulb; TeO, optic tectum; T, thalamic region; Ce, cerebellum; MO, medulla oblongata.

1.4 Circadian regulation of sleep

The circadian regulation of sleep generates an internal sleep/wake rhythmicity synchronized to the external environment (e.g. light and availability of food) and ensures that sleep occurs at appropriate times (Golombek and Rosenstein, 2010). In mammals, the circadian rhythmicity primarily arises from the central circadian clock generated by the SCN in the anterior hypothalamus (Klein et al., 1991). The SCN receives direct innervations from the eye via the retinohypothalamic tract (Morin and Allen, 2006). In a natural environment, it generates a ~24-hour circadian clock according to the Earth's rotation (Panda et al., 2002).

In 1971, the identification of the *period* gene in a forward genetic screen in *Drosophila* by Ronald Konopka and Seymour Benzer paved the foundations for the molecular basis of circadian clock in animals (Konopka and Benzer, 1971). Subsequent characterization of the circadian clock demonstrated that the self-sustained molecular oscillator is mediated by a series of interconnected transcriptional autoregulatory feedback loops conserved throughout evolution (Reppert and Weaver, 2001). The detailed components of the molecular oscillator have been extensively reviewed (Lowrey and Takahashi, 2004; Ko and Takahashi, 2006; Partch et al., 2014) and depicted in Figure 1.2. In mammals, the circadian locomotor cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1) proteins act as major transcriptional activators by forming a heterodimer that promotes transcription of *Period* (*Per*) and *Cryptochrome* (C*ry*) (Figure 1.2, Gekakis et al., 1998; Kume et al., 1999). Following translation, PER and CRY proteins are phosphorylated by casein kinase 1 ε and δ (CK1). The phosphorylated PER and CRY dimerize and translocate back to the nucleus to repress the transcription of

Bmal1 and *Clock*, hence creating a negative feedback loop (Akashi et al., 2002; Lee et al., 2009). An adjoining negative regulatory loop induced by CLOCK-BMAL1 heterodimers is the transcription of retinoic acid-related orphan nuclear receptors, *Rev-erbα*. Once translated, REV-ERBα protein inhibits transcription of *Bmal1* by binding retinoic acid-related orphan receptor response elements (ROREs) in *Bmal1* promoter (Triqueneaux et al., 2004; Akashi and Takumi, 2005; Guillaumond et al., 2005).

Each SCN neuron can function as a cell-autonomous oscillator (Welsh et al., 1995). Synchronization among the SCN cells is essential to produce a robust circadian rhythmicity. Synchronization within the SCN is achieved by the release of several neurotransmitters in the SCN, including γ-aminobutyric acid (GABA) (Liu and Reppert, 2000), vasoactive intestinal polypeptide (VIP) (Aton et al., 2005; Maywood et al., 2006), gastrin-releasing peptide (GRP) (McArthur et al., 2000; Gamble et al., 2007), and vasopressin (AVP) (Maywood et al., 2011), as well as the presence of gap junctions that mediate electrical synchronization among SCN neurons (Colwell, 2000; Long et al., 2005).

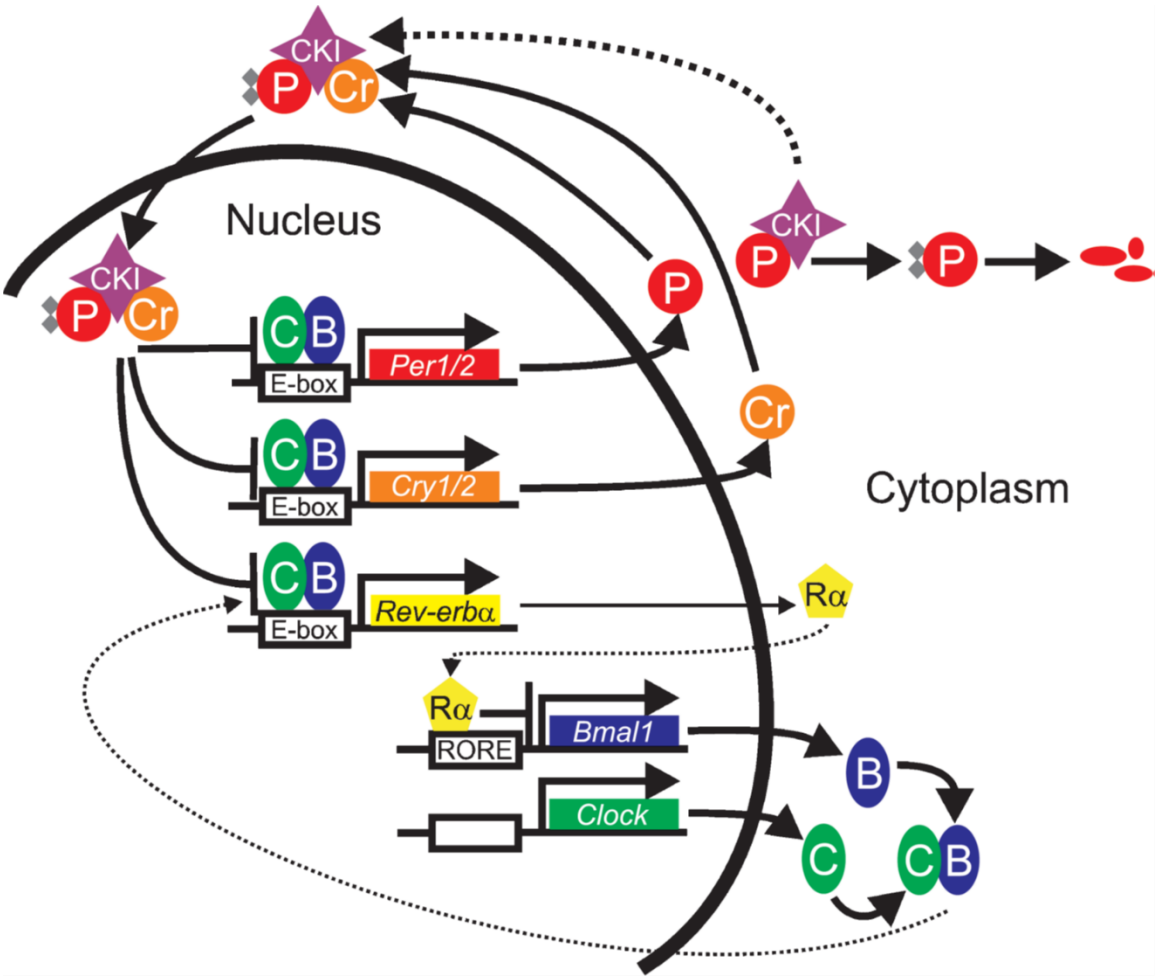


Figure 1.2. Diagram of the mammalian core circadian clock. Abbreviations: PER (P), CRY (Cr), BMAL1 (B), CLOCK (C), CKIε (CKI), REV-ERBα (Rα). Gray diamonds represent phosphorylation. (Modified figure from Lowrey and Takahashi, 2004)

1.5 SCN projections to sleep-wake centers

The major efferent pathway from the SCN is summarized in Figure 1.3. The SCN predominantly projects to the subparaventricular zone (SPZ), which is sub-divided into a ventral part (vSPZ) located above the SCN, and a dorsal part (dSPZ) located below the hypothalamic paraventicular nucleus (PVN) in mammals (Figure 1.3, Watts and Swanson, 1987; Watts et al., 1987). Lesions to the vSPZ disrupt the circadian regulation of sleep-

wake and locomotor activity, while lesions of the dSPZ impair circadian rhythm of body temperature, suggesting that efferent projection from vSPZ is essential for relaying circadian signals to the sleep-wake centers (Lu et al., 2001; Moore and Danchenko, 2002; Abrahamson and Moore, 2006).

The SCN and vSPZ show some direct projection to the sleep and wake promoting centers (Sun et al., 2000; Chou et al., 2002; Yoshida et al., 2006), although most of SCN projection to the sleep and wake promoting center is mediated through the dorsalmedial hypothalamus (DMH), a major target of the vSPZ (Watts, 1991). Lesion of the DMH impairs circadian rhythmicity of sleep-wake cycles and locomotor activity (Chou et al., 2003). DMH then sends mostly GABAergic innervation to the VLPO, and glutamatergic and thyrotropin-releasing hormone projections to the Hcrt neurons in the lateral hypothalamus (Thompson et al., 1996; Chou et al., 2003). The Hcrt neurons send excitatory outputs to the entire monoaminergic arousal system, including the histaminerginc TMN, serotoniergic DR, noradrenergic LC, and cholingneric neurons of the PPT and basal forebrain (Peyron et al., 1998; Siegel, 2004). These areas then relay SCN messages throughout the rest of the brain and peripheral organs that, in turn, manifest circadian rhythm in behavior output such as sleep/wake cycle and locomotor activity (Saper et al., 2005b).

The SCN also projects to the pineal gland via a multi-synaptic pathway and regulates the level of melatonin, a pineal hormone that signals darkness and promotes sleep in diurnal animals (Hardeland, 2008; Fisher et al., 2013; Gandhi et al., 2015). Circadian signals from the SCN are transmitted to the paraventricular nuclei of the hypothalamus (PVH), intermediolateral nucleus of the spinal cord, superior cervical

ganglion (SCG), and then finally the pineal gland (Borjigin et al., 2012). The SCN uses a combination of daytime inhibitory and nighttime stimulatory signals to control the daily rhythm of pineal melatonin synthesis (Perreau-Lenz et al., 2003). At night, the glutamatergic output from the SCN innervating the PVH stimulates the release of norepinephrine from the SCG, which acts on beta-adrenergic receptors in the pineal gland to increase melatonin synthesis (Perreau-Lenz et al., 2004). During the day, GABAergic output from the SCN to PVH inhibits melatonin synthesis (Kalsbeek et al., 2000).

1.6 SCN output factors affecting sleep/wake behavior

In addition to the synaptic transmission and neuroendocrine regulation of sleep/wake cycle, the SCN also regulates behavioral state by diffusible factors (Silver et al., 1996). In 1996, Silver et al. discovered that preventing axonal outgrowth from SCN grafts using a semipermeable polymeric capsule restored circadian rhythmicity in animals whose own SCN was ablated.

So far, four candidate molecules expressed in the SCN have been discovered and proposed as circadian output molecules that regulate sleep/wake cycles or locomotor activity: transforming growth factor-α (TGF-α) (Kramer et al., 2001), Prokineticin 2 (PROK2) (Cheng et al., 2002), AVP (Tousson and Meissl, 2004; Li et al., 2009a), and cardiotrophin-like cytokine (CLC) (Kraves and Weitz, 2006).

TGF-α is expressed in a rhythmic fashion in the astrocytes of the SCN with peak levels during the day (Kramer et al., 2001; Li et al., 2002). Infusion of TGF-α inhibits locomotor activity and disrupts circadian sleep-wake cycle, and the effect is mediated by epidermal growth factor receptor (EGFR) in the SPZ. Mice with a hypomorphic EGF

receptor mutation show increased daytime locomotor activity, suggesting TGF-α may function as a SCN inhibitor of locomotion (Kramer et al., 2001). EGFR's conservative role in sleep regulation has also been described in *C. elegans*, and *Drosophila* (Foltenyi et al., 2007; Van Buskirk and Sternberg, 2007).

AVP concentration in the cerebrospinal fluid (Reppert et al., 1981) and in the SCN show circadian rhythmicity with a peak at early light phase (Tominaga et al., 1992). Its expression in the SCN is activated by the CLOCK-BMAL1 complex (Jin et al., 1999). AVP also has a well-established role in circadian regulation of hormone release (Kalsbeek et al., 2010). Neither central infusion of vasopressin (Albers et al., 1984), vasopressin receptor antagonists (Stoynev and Nagai, 1996), nor loss of AVP (Groblewski et al., 1981) changed circadian rhythms in locomotor activity rhythms in rodents. However, mice deficient in vasopressin receptor V1a, which is predominantly expressed in the SCN, exhibited attenuated circadian rhythmicity of locomotor activity (Li et al., 2009a).

PROK2's role in regulating sleep/wake behavior is described in detail in chapter 2. Briefly, PROK2 expression in the SCN exhibits circadian oscillation with high mRNA level during the day and low level at night (Cheng et al., 2002). Infusion of PROK2 at night suppresses nocturnal wheel-running activity (Cheng et al., 2002). Prok2 mutants show attenuated circadian rhythmicity (Li et al., 2006) and decreased total sleep (Hu et al., 2007).

CLC is expressed in a subpopulation of SCN vasopressin neurons and its expression level peaks during the late day in mice (Kraves and Weitz, 2006). Infusion of

CLC in the dark phase blocks locomotor activity, while neutralizing CLC receptor with antibodies increased locomotor activity in the late day (Kraves and Weitz, 2006).

To date, diffusible factors that promote arousal and activity have not been identified.

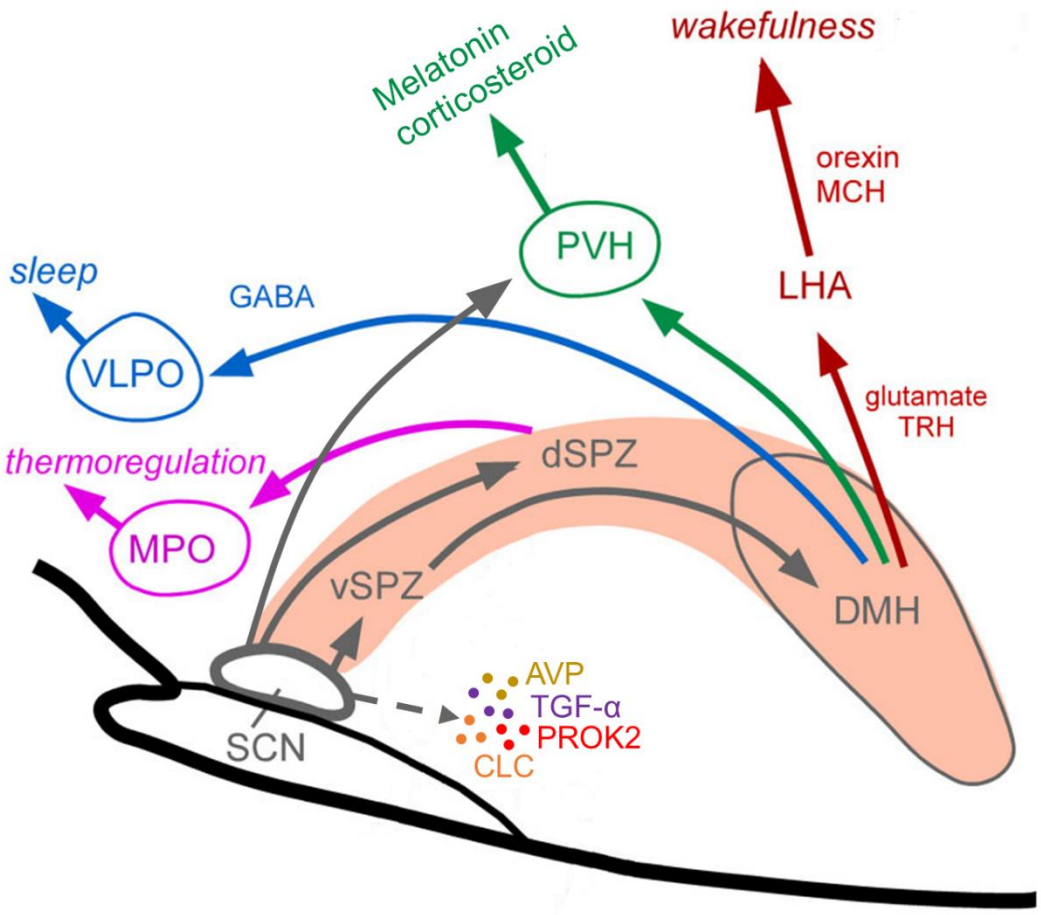


Figure 1.3. A summary diagram to illustrate the major pathways that translate the output from the brain's biological clock (the SCN) into circadian rhythms of sleep/wake cycle. (Modified figure from Saper et al., 2005)

1.7 Regulation of sleep in diurnal vs nocturnal animals

Both diurnal and nocturnal animals have identical central clock and sleep-wake components (Caldelas et al., 2003; Saper et al., 2005c; Challet, 2007). Neuronal activity of the SCN peaks during the day in both diurnal and nocturnal animals, and the clock gene expression in the SCN show similar patterns relative to light and dark phases (Sato and Kawamura, 1984; Nunez et al., 1999; Dardente et al., 2002), suggesting there is a reversal of the circadian circuitry controlling overt rhythm downstream of the central clock. However, the neural basis for the inversion remains unclear. vSPZ has been suggested as a candidate site of circadian signal inversion as daily neuronal activity pattern in the vSPZ differs in diurnal and nocturnal rats (Schwartz et al., 2004). Melatonin, a molecule that is expressed in the darkness in both diurnal and nocturnal animals (Hardeland, 2008), has been shown to promote sleep in diurnal animals (Goldstein and Pavel, 1981; Mintz et al., 1998; Zhdanova et al., 2001, 2002; Gandhi et al., 2015) but promote wakefulness (Mendelson et al., 1980) or have no effect on sleep in nocturnal rodents (Tobler et al., 1994), suggesting circadian molecules may function differently in diurnal vs nocturnal animals.

Most studies of sleep regulation have focused on nocturnal rodents, leading to an under-representation of diurnal animals in the field. In addition, the complexity of the mammalian brains have partly hindered the elucidation of genetic and neural mechanisms that regulate sleep. To better understand the genetic and neural mechanism of sleep regulatory circuitry in humans, a simple diurnal vertebrate model is essential.

1.8 Using larval zebrafish as a model for studying behavior

Larval zebrafish has recently emerged as a popular model organism to study genetic and neuronal control of behavior, attributed by several unique properties of larval zebrafish (Fetcho and Liu, 1998). First, shortly after larval zebrafish hatch, they show rapid responses to a range of sensory cues and execute extended behavioral response to changes in environment (Guo, 2004; Kalueff et al., 2013). Second, mammalian and zebrafish brains are morphologically and molecularly conserved, suggesting that similar neural circuits may regulate zebrafish and mammalian behaviors. However, the complex neuro-regulatory circuits are much simpler in larval zebrafish (Ekström, 1994; Ma, 1994a, 1994b; Wullimann et al., 1996; Rink and Wullimann, 2001; Mueller et al., 2004; Prober et al., 2006). Third, zebrafish larvae are transparent, which allows direct observation and manipulation of neurons activity by optical and optogenetic tools in live animals (Liu and Fetcho, 1999; Higashijima et al., 2003; Portugues et al., 2013). Fourth, zebrafish breed in large numbers, enabling high throughput genetic and pharmacological experiments that are much more difficult to perform with other vertebrate models (Brockerhoff et al., 1995; Gross et al., 2005; Muto et al., 2005; Kokel et al., 2010; Rihel et al., 2010a; Wolman et al., 2011). Fifth, zebrafish behaviors can be studied and manipulated by dissolving small molecules in water, which can be taken up through the gills or absorbed through the skin (Goldsmith, 2004; Peterson and Fishman, 2004; Rihel et al., 2010a). Like other model organisms, the zebrafish genome is fully characterized, and recent development in genome editing tools such as TALEN and CRISPR allows easy manipulation of the zebrafish genome (Sander et al., 2011b; Hwang et al., 2013; Bedell and Ekker, 2015). In addition, zebrafish larvae are sustained by a yolk sac for the first week of development,

eliminating feeding as a potential confounding variable in behavior experiments (Prober et al., 2006). Larval zebrafish combine the relative simplicity of *Drosophila* and *C. elegans* with behavioral complexity and brain structures that partially resemble mammals, making it an attractive model to study behavior. In fact, it has been widely used in neurogenetic analysis of behavior including locomotion (Budick and O'Malley, 2000), sensory response (Granato et al., 1996; Muto et al., 2005; Emran et al., 2007), escape and avoidance (Colwill and Creton, 2011; Pelkowski et al., 2011), circadian and sleep (Zhdanova et al., 2001; Hurd and Cahill, 2002; Prober et al., 2006; Gandhi et al., 2015), reward (Bretaud et al., 2007), and learning and memory assays (Best et al., 2008; Roberts et al., 2013), demonstrating that zebrafish larvae can be a powerful tool for elucidating neuronal pathways underlying behavior.

1.9 Sleep in larval zebrafish

Larval zebrafish meet all three behavior criteria for sleep.

A prolonged period of quiescence occurring in specific periods of the circadian cycle - Larval zebrafish display robust sleep/wake behavior starting on the fourth day of development (Hurd and Cahill, 2002; Prober et al., 2006). Like humans, zebrafish larvae are diurnal and exhibit peak swim activity during the day and sleep-state at night. When transferred to constant dark conditions, larval zebrafish maintain circadian oscillation in locomotor activity, suggesting that zebrafish larvae have an endogenous circadian rhythm controlling behavior (Hurd et al., 1998; Hurd and Cahill, 2002). The core molecular machinery of the mammalian circadian clock is also conserved in zebrafish (Vatine et al., 2011). However, some differences exist between zebrafish larvae and mammals. The

zebrafish peripheral clock can be directly entrained by light, likely due to its transparency (Whitmore et al., 2000). In addition, zebrafish larvae raised in constant light conditions lack overt behavior rhythmicity and oscillation of core clock genes, enabling study of sleep behavior in the absence of circadian influence (Hurd and Cahill, 2002; Prober et al., 2006; Gandhi et al., 2015).

Elevated sensory arousal threshold – zebrafish larvae in quiescent periods have increased arousal threshold. They show reduced responsiveness to mechanical stimuli and sudden change in light intensity (Zhdanova et al., 2001; Prober et al., 2006). A sleep-like state is defined as at least one minute of inactivity in zebrafish larvae because one minute or longer inactivity is associated with an increase in arousal threshold (Prober et al., 2006).

Homeostatic regulation – zebrafish larvae deprived of sleep at night by a mechanical shaker show a subsequent decrease in locomotor activity accompanied by an increased in arousal threshold response to mechanical stimulus (Zhdanova et al., 2001).

Anatomically, zebrafish larvae possess homologous structures that are known to regulate mammalian sleep (Figure 1.1), including the noradrenergic locus coeruleus (Guo et al., 1999), the histaminergic tuberomammillary nucleus (Kaslin and Panula, 2001), the serotonergic dorsal raphe (McLean and Fetcho, 2004), the dopaminergic ventral tegmental area and substantia nigra (Ryu et al., 2007; Sallinen et al., 2009), the galaninerigc ventrolateral preoptic area (Podlasz et al., 2012), the hypocretinergic and the MCH expressing lateral hypothalamus (Prober et al., 2006; Berman et al., 2009), and cholinergic regions (Yokogawa et al., 2007; Hong et al., 2013).

Many known mammalian sleep regulators are also conserved in zebrafish larvae, but expressed in much fewer cells. For example, loss of neuropeptide Hcrt or its receptor causes narcolepsy in humans, dogs, and mice, characterized by fragmented sleep, daytime sleepiness, and cataplexy (Zeitzer et al., 2006; Sakurai, 2007). Injection of Hcrt peptide promotes locomotor activity and inhibits sleep in rodents (Bourgin et al., 2000). Similarly, Hcrt overexpression in zebrafish larvae promotes locomotor activity and inhibits sleep (Prober et al., 2006). In addition, like mammals, zebrafish Hcrt neurons project to aminergic and cholinergic neurons (Kaslin et al., 2004; Prober et al., 2006; Sakurai, 2007). However, unlike the thousands of Hcrt neurons in the mammalian brain, zebrafish larvae only have ~10 Hcrt neurons, making it much simpler to study (Prober et al., 2006). In addition, melatonin, a pineal hormone that has been shown to promote sleep in diurnal mammals, has also been recently demonstrated to promote sleep and is required for circadian regulation of sleep in zebrafish larvae (Gandhi et al., 2015).

Another distinct advantage of studying sleep in zebrafish larvae is its amenability for pharmacological studies. Zebrafish larvae lack a mature blood brain barrier and can take up small molecules dissolved in water by gills or through the skin (Peterson and Fishman, 2004; Zon and Peterson, 2005; Fleming et al., 2013). Several studies have shown that the mammalian sleep/wake neuropharmacology is conserved in zebrafish larvae, suggesting that similar mechanisms may regulate sleep in larval zebrafish and mammals (Zhdanova et al., 2001; Renier et al., 2007; Rihel et al., 2010a).

This thesis attempts to use the advantages of larval zebrafish to answer unsolved questions in regulation of sleep behavior in diurnal vertebrate and expand the existing repertoire of genetic tools to manipulate neuronal activity in zebrafish larvae.

1.10 Outline of the thesis

Chapter 2 focuses on the use of larval zebrafish to characterize the role of Prok2 in sleep regulation in a diurnal vertebrate, attempting to answer the question whether studies of circadian output factors in nocturnal animals can be directly translated to diurnal animals. I show the first genetic gain of function experiment in Prok2 and that Prok2 overexpression is sufficient to induce changes in sleep behavior in a light dependent manner via Prok receptor 2. Larval zebrafish with a Prok2 loss of function mutation confirm that Prok2 is also necessary for normal sleep behavior during the day in larval zebrafish, but not for sleep/wake circadian rhythmicity in free running conditions. Generation of loss of function mutants is summarized in Appendix. I then compare the functional difference of Prok2 in diurnal vs nocturnal animals and propose larval zebrafish as an alternative for studying circadian regulation of sleep in humans. Chapter 3 focuses on adapting genetic tools to manipulate neuronal activity in larval zebrafish and a novel method of using them. I show that three transient receptor potential (TRP) channels, TRPV1, TRPM8, and TRPA1, can inducibly activate specific populations of neurons in larval zebrafish by using their appropriate agonists. At high concentration of its agonist, TRPV1 could also rapidly ablate cells. Combinations of these channels enable the manipulation of multiple neuronal groups in larval zebrafish and expand the variety of behavior experiments that could be performed. In chapter 4, I conclude the dissertation by summarizing the findings of the previous chapters and proposing future experiments for understand Prok2 regulation of sleep/wake cycles, including using TRP channels.

CHAPTER 2

LIGHT DEPENDENT REGULATION OF SLEEP/WAKE STATES BY PROKINETICIN 2 IN ZEBRAFISH

2.1 Abstract

The circadian clock regulates diverse biological processes to ensure they occur at appropriate times during the 24 hour day/night cycle. In mammals, the suprachiasmatic nucleus (SCN) acts as the master circadian pacemaker to regulate circadian rhythms in tissues and cells throughout the animal. While the circadian clock mechanism has been described at the molecular level, it remains poorly understood how circadian information is transmitted from the SCN to other tissues, or how the circadian clock regulates physiological and behavioral processes such as sleep. Several secreted proteins have been proposed to act as circadian output molecules that transmit this circadian information. However, these proteins have primarily been studied in nocturnal rodents, and their functions in diurnal vertebrate animals are largely unknown. Here we describe the role of one of these factors, prokineticin 2 (Prok2), in regulating sleep/wake behaviors in the zebrafish, a diurnal vertebrate. We show that *prok2* is exclusively expressed in the larval zebrafish ventral hypothalamus, a potential homolog of the mammalian SCN. We found that genetic overexpression of *prok2* both increases sleep during the day and decreases sleep at night, in a manner that depends on lighting conditions and not the circadian clock. These phenotypes require Prok receptor 2, which is expressed in neurons associated with arousal, and not Prok receptor 1. Finally, we show that *prok2* mutant larvae exhibit increased locomotor activity and decreased sleep during the day in light/dark conditions, but have normal circadian patterns of locomotor activity and sleep in free-running conditions. Our results indicate that Prok2 is not required for the circadian regulation of sleep in zebrafish, and rather suggest that Prok2 acts as a homeostatic regulator of sleep/wake states.

2.2 Introduction

Sleep is a conserved behavior that is observed across the animal kingdom but the mechanisms that regulate sleep remain poorly understood (Cirelli, 2009). The classical two-process model of sleep regulation (Borbély, 1982) postulates that sleep is regulated by two processes. First, a homeostatic process that responds to internal cues of sleep need increases during wakefulness and dissipates during sleep. Second, a circadian process responds to environmental cues to ensure that sleep occurs at the appropriate time during the 24-hour day/night cycle. While the circadian clock mechanism has been described in detail at the molecular level (reviewed in Partch et al., 2014), it remains poorly understood how the circadian clock regulates physiology and behavior, or how circadian information is transmitted from master circadian pacemakers such as the mammalian SCN to other tissues. Several secreted factors, including arginine vasopressin (AVP) (Brown and Nunez, 1989; Li et al., 2009a), melatonin (Gandhi et al., 2015), cardiotrophin-like cytokine (CLC) (Kraves and Weitz, 2006), transforming growth factor alpha (TGF-α) (Kramer et al., 2001), and prokineticin 2 (Prok2) (Cheng et al., 2002) have been proposed to act as factors that transmit circadian information to regulate behavior and physiology. This hypothesis is based on the observation that the circadian clock regulates the expression of these factors, and that gain or loss of these factors affects processes that are regulated by the circadian clock. However, these factors have primarily been studied in nocturnal rodents, and their functions in diurnal vertebrate animals are largely unknown.

Prok2 is a cysteine rich secreted peptide that has diverse biological functions (LeCouter et al., 2001; Li et al., 2001, 2009b; Ng et al., 2005; Gardiner et al., 2010). Loss

of *prok2* results in Kallmann syndrome in humans and rodents, which is characterized by hypogonadism and anosmia (Dodé et al., 2006; Matsumoto et al., 2006; Pitteloud et al., 2007; Cole et al., 2008). A role for Prok2 in regulating sleep is also supported by several studies in nocturnal rodents. First, *prok2* mRNA is highly expressed in the rodent SCN and oscillates with circadian rhythmicity, with high expression levels during the day and low levels at night (Cheng et al., 2002; Ji and Li, 2009; Noonin et al., 2013). This expression is regulated by both the endogenous circadian clock and by light (Cheng et al., 2005; Ren et al., 2011). Consistent with a role for Prok2 as an SCN output factor, one of its two receptors, Prok receptor 2 (Prokr2), is expressed in many SCN target nuclei in the brain (Cheng et al., 2006; Masumoto et al., 2006; Ji and Li, 2009). Second, intracerebroventricular (ICV) injection of Prok2 protein in nocturnal rodents at night, when they are primarily awake, suppresses locomotor activity, while mice that lack *prok2* or *prokr2* exhibit attenuated rhythmicity of several processes regulated by the circadian clock, including locomotor activity, thermoregulation, and circulating corticosteroid and glucose levels (Cheng et al., 2002; Li et al., 2006; Prosser et al., 2007). In addition, loss of *prok2* reduces total sleep and sleep recovery compared to baseline after sleep deprivation (Hu et al., 2007). In contrast to studies using nocturnal rodents, a recent study of humans with a *prok2* loss of function mutation found no abnormalities in several processes regulated by the circadian clock, including levels of circulating melatonin and cortisol, or body temperature, but did find impaired psychomotor vigilance task performance (Balasubramanian et al., 2014). These results suggest that molecules that have been identified as circadian output factors such as Prok2 nocturnal animals may not

act as such in diurnal animals like humans. As a result, it is important to study the functions of these factors in diurnal model organisms.

Several studies suggest that circadian clock output factors may indeed have different functions in nocturnal and diurnal animals. For example, the hormone melatonin is produced at night in both nocturnal and diurnal animals, and is thus present during the circadian phase when diurnal animals are primarily asleep and nocturnal animals are primarily awake (Korf et al., 1998; Kazimi and Cahill, 1999). Melatonin has been shown to promote sleep in several diurnal species (Goldstein and Pavel, 1981; Mintz et al., 1998; Zhdanova et al., 2001, 2002; Zhdanova, 2005) and is required for the circadian regulation of sleep in the diurnal zebrafish (Gandhi et al., 2015), whereas melatonin has been shown to be wake-promoting (Mendelson et al., 1980) or to have no effect on sleep (Tobler et al., 1994) in nocturnal animals. Based on these observations, melatonin is thought to act as a hormonal signal for processes specific to the dark phase of the circadian cycle (Turek and Gillette, 2004), with different functions in nocturnal and diurnal animals. This distinction highlights the need to study circadian clock output factors in diurnal animal models in order to understand how they may act in humans.

Recent studies have shown significant conservation of sleep/wake behaviors in simple and genetically tractable model organisms such as C. *elegans*, *Drosophila*, and *Danio rerio* (zebrafish) (Shaw et al., 2000; Raizen et al., 2008; Zimmerman et al., 2008; Singh et al., 2011). The zebrafish is a diurnal vertebrate that exhibits behavioral, genetic, anatomical, and pharmacological conservation of mammalian sleep biology (Zhdanova et al., 2001; Kaslin et al., 2004; Faraco et al., 2006; Prober et al., 2006; Renier et al., 2007; Yokogawa et al., 2007; Appelbaum et al., 2009; Rihel et al., 2010a). Because the

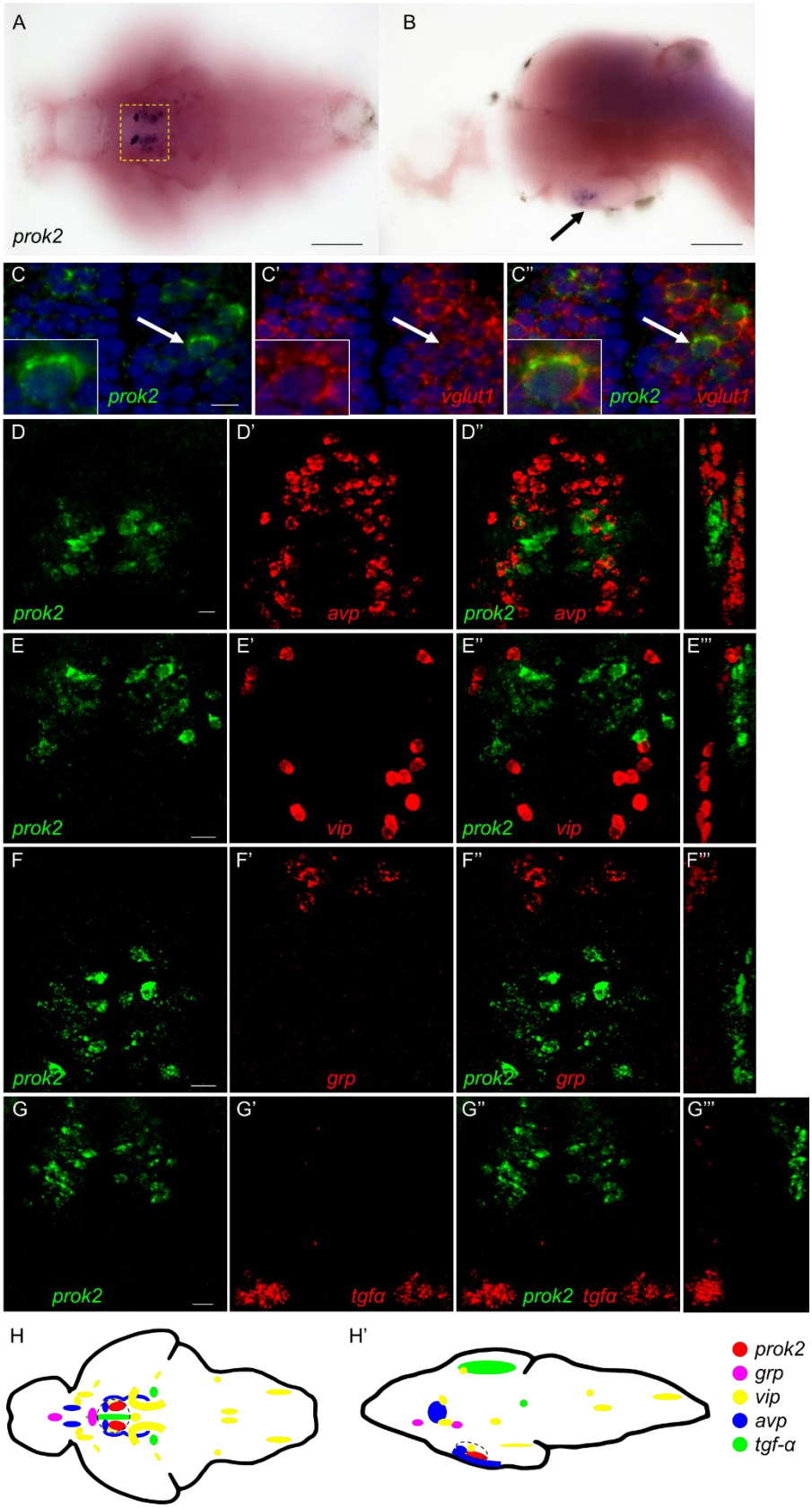
zebrafish is diurnal, it may be a more relevant model than nocturnal rodents for how the circadian clock regulates sleep in humans. To understand the function of a putative circadian output factor in regulating sleep in a diurnal vertebrate, here we analyze the effects of Prok2 gain- and loss-of-function perturbations on sleep/wake behaviors in larval zebrafish.

2.3 *prok2* is expressed in the larval zebrafish ventral hypothalamus

Previous studies identified a single zebrafish orthologue of *prok2* (Ayari et al., 2010), but its expression pattern in larvae has not been described. Using *in situ* hybridization (ISH), we found that *prok2* is exclusively expressed in a bilateral cluster of neurons in the ventral hypothalamus on the fifth day of development (Figures 2.1A, B), a similar brain region to the mammalian SCN. Each cluster consists of ~10 neurons and is located adjacent to the optic chiasm (Wullimann and Knipp, 2000). Double-fluorescent ISH (FISH) revealed that all *prok2*-expressing neurons express *vesicular glutamate transporter 1*, and thus are glutamatergic (Figures 2.1C-2.1C’’’). A previous study reported more widespread *prok2* expression in the adult zebrafish brain (Ayari et al., 2010). Furthermore, in rodents *prok2* is expressed in many brain regions in addition to the SCN, including the nucleus accumbens, globus pallidus, medial amygdala, medial preoptic area, arcuate nucleus, and olfactory bulb (Cheng et al., 2002, 2006). Thus, either *prok2* expression is more restricted in larval zebrafish than in rodents and adult zebrafish, or expression in regions other than the ventral hypothalamus is too weak to detect at larval stages. In either case, our data suggest that *prok2* is predominantly expressed in the ventral hypothalamus in zebrafish larvae. This restricted expression pattern suggests that

zebrafish larvae may provide a simpler system to study the role of Prok2 in regulating sleep compared to adult zebrafish and rodents.

Because *prok2* is highly expressed in the rodent SCN and has been proposed to be a circadian output molecule, we compared *prok2* expression with the zebrafish orthologues of other mammalian SCN markers using double FISH. In mammals, *vasoactive intestinal peptide* (*vip*)- and *gastrin-releasing peptide* (*grp*)-expressing neurons are found in the core of SCN, while *arginine vasopressin* (*avp*)-expressing neurons are found in the shell of SCN (Masumoto et al., 2006). *transforming growth factor alpha* (*tgfα*) is expressed throughout the mammalian SCN but is found more abundantly in the core (Van Der Zee et al., 2005). *prok2* is expressed in both the dorsomedial (shell) and ventrolateral (core) SCN, and more than 50% of the *prok2*-expressing neurons also express *vip*, *grp*, or *avp* (Masumoto et al., 2006). We observed that *avp* is expressed in the ventral hypothalamus just rostral to *prok2*, as well as in cells ventral to *prok2* (Figures 2.1D-2.1D’’’). *avp* is also expressed in bilateral clusters in the neurosecretory preoptic area (NPO) (Figures 2.1H-2.1H’), which corresponds to the mammalian paraventricular nucleus of the hypothalamus (Herget and Ryu, 2015). *vip* is expressed in a small bilateral cluster of cells just rostral to *prok2*, as well as in several discrete nuclei in the diencephalon, midbrain, and hindbrain (Figures 2.1E-2.1E’’’, 2.1H-2.1H’). *grp* is expressed in two clusters of cells near the NPO in close proximity to *avp*-expressing neurons, but rostral and dorsal relative to *prok2*-expressing neurons (Figures 2.1E-2.1E’’’, 2.1H-2.1H’). However, we did not observe coexpression of *prok2* with *avp*, *grp*, or *vip*. *tgfα* is expressed in a bilateral cluster of cells in the midbrain (Figures 2.1G-2.1G”’) and in cells along the diencephalic ventricle (Figures 2.1H-2.1H’’’), but is not


A
B
prok2
C
C'
C''
prok2
vglut1
prok2
vglut1
D
D'
D''
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G'''
prok2
tgfα
prok2
tgfα
H
H'
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grp
vip
avp
tgf-α

Figure 2.1. *prok2* expression in larval zebrafish brain. (A, B) *prok2* mRNA is expressed in a bilaterally symmetric cluster of neurons in the ventral hypothalamus that each contains about 10 neurons at 5 dpf (A, ventral view; B, lateral view; anterior at left). Dashed box indicates the ventral hypothalamic region shown in (C). (C) A 0.46 μm confocal section shows that *prok2*-expressing neurons (green) coexpress *vesicular glutamate transporter 1* (*vglut1*) (red). DAPI nuclear staining is shown in blue. One representative cell, indicated by arrow, is enlarged in inset. C''' shows the orthogonal view of (C'') at the red line. Blue line in C''' indicates the z position of C''. Anterior at top. (D-G) Confocal projections show *prok2* is expressed caudal to *avp*- (D) and *vip*- (E) expressing cells in the ventral hypothalamus. *grp* (F) and tgf*α* (G) are not expressed in the ventral hypothalamus. D''', E''', F''', and G''' show the orthogonal projection of each confocal stack. Anterior at top. (H) Schematic drawing illustrates relative position of *prok2*, *avp*, *vip*, *grp*, and *tgfα* expressing neurons in larval zebrafish brain at 5 dpf (A, ventral view; B, lateral view, anterior at left). Dashed circles indicate the putative SCN. Schematics include expression in brain regions not shown in (C-G). Scale bars: A-B, 100 μm; C-G, 10 μm.

expressed in the ventral hypothalamic region. Thus, three out of four mammalian SCN markers are expressed in the ventral hypothalamus in close proximity to the optic chiasm in larval zebrafish, suggesting that this brain region could be a homolog of the mammalian SCN.

2.4 *prok2* expression does not oscillate in a circadian manner in larval zebrafish

Using ISH and quantitative reverse-transcription PCR (qRT-PCR), *prok2* transcript level was previously reported to oscillate in a circadian manner in rodents (Cheng et al., 2002; Noonin et al., 2013). To test whether *prok2* expression oscillates in zebrafish larvae, we isolated total RNA from larvae at 6 days post-fertilization (dpf) at 4 hour intervals over the course of 24 hours, and measured *prok2* transcript level using qRT-PCR. In contrast to rodents, we did not observe a significant change in *prok2* expression level (Figure S2.1A), whereas expression of the circadian clock gene *period3* oscillated in a circadian manner as expected (Delaunay et al., 2000). Consistent with this

result, we did not observe changes in *prok2* expression using FISH at multiple circadian time points (Figure S2.1B, 2.1C). In contrast to these results, a previous study using qRT-PCR suggested that *prok2* transcript may be elevated at the transition from dark to light phase in adult zebrafish (Noonin et al., 2013). However, *prok2* is expressed in several brain regions in adult zebrafish and it was not determined where this expression change occurs. Thus, in contrast to rodents, our results indicate that *prok2* expression does not oscillate in a circadian manner in zebrafish larvae.

2.5 Prok2 overexpression inhibits locomotor activity and promotes sleep during the day in zebrafish larvae

A previous study showed that ICV infusion of Prok2 protein in nocturnal rats at night suppresses locomotor activity. Taken together with the observation that *prok2* expression levels in the SCN are high during the day, when nocturnal rodents are primarily asleep, this result can be interpreted to mean that Prok2 normally acts as a signal to promote day-specific behaviors. If this "day-specific" hypothesis is correct, Prok2 overexpression should promote locomotor activity and/or inhibit sleep in diurnal animals. Alternatively, Prok2 may act as a signal for behaviors specific for the inactive phase of the 24 hour circadian period. If this "inactive phase" hypothesis is correct, Prok2 overexpression should promote sleep in diurnal animals. To distinguish between these possibilities, we generated transgenic zebrafish in which a heat shock inducible promoter regulates expression of the zebrafish Prok2 orthologue (*Tg(hsp:Prok2))* and monitored the effects of Prok2 overexpression on sleep/wake behaviors using a high throughput videotracking assay (Prober et al., 2006). We did not observe any difference in the

locomotor activity or sleep between *Tg*(*hsp:Prok2)* larvae and their wild type (WT) siblings before Prok2 overexpression (Figures 2.2A-2F). However, after heat shock, *Tg(hsp:Prok2)* larvae showed a dramatic decrease in locomotor activity and increase in sleep during the day. This phenotype was caused by an increase in both the number (Figure 2.2G) and length (Figure 2.2I) of sleep bouts, with a corresponding decrease in the length of wake bouts (Figure 2.2K). This result suggests that Prok2 acts as a signal to promote inactivity and/or sleep, rather than as a signal for day-specific behaviors, in zebrafish larvae.

However, we also observed that Prok2 overexpression increased locomotor activity and decreased sleep at night (Figures 2.2A, 2.2B, 2.2D, 2.2F). This phenotype was caused by a decrease in the length of sleep bouts (Figure 2.2J), with a non-significant trend towards longer wake bouts (Figure 2.2L), and is in contrast with a simple "inactive phase" role for Prok2. Similarly, ICV infusion of Prok2 at night in nocturnal rodents decreased locomotor activity at night but also increased locomotor activity the following day (Cheng et al., 2002). The opposite phenotype during the following day was hypothesized to be an indirect consequence of Prok2 signaling at night, perhaps due to desensitization of Prok2 receptors or a "rebound" in activity following a night of decreased activity. However, it is also possible that Prok2 signaling directly promotes opposite sleep/wake phenotypes during the day and night, thus acting as a homeostatic regulator instead of a simple inactivity inducer. To distinguish between these hypotheses, we repeated the behavioral experiment but performed the heat shock at night. Following a short recovery period after the heat shock, Prok2-overexpressing larvae exhibited increased locomotor activity and decreased sleep at night (Figures 2.3A-2.3F).

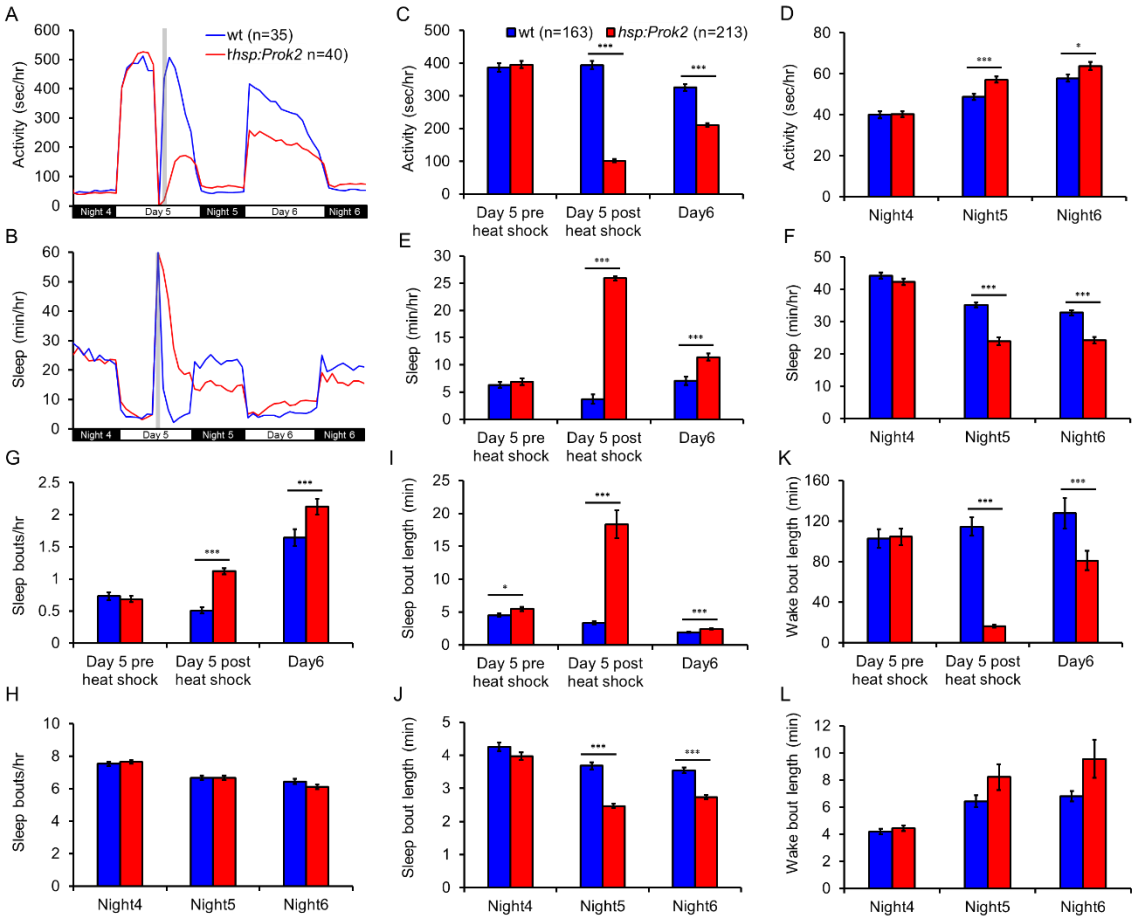

Figure2. 2. Prok2 overexpression promotes sleep during the day and activity at night following daytime heat shock. Heat shock (grey bar) during the day decreases locomotor activity during the day (A, C) and increases locomotor activity at night (A, D) for *Tg(hsp:Prok2)* larvae (red) compared to their non-transgenic siblings (blue). Prok2 overexpression also increases sleep during the day (B, E) and decreases sleep at night (B, F). These phenotypes were observed for over 36 hours following heat shock. Increased daytime sleep due to Prok2 overexpression results from an increase in the number (G) and length (I) of sleep bouts, with a corresponding decrease in the length of wake bouts (K). Decreased sleep at night due to Prok2 overexpression results from a decrease in the length of sleep bouts (J). Data from one representative experiment (A, B) and five experiments combined (C-L) are shown. (C-L) show mean ± SEM. n indicates number of larvae analyzed. *$p < 0.05$, ***$p < 0.001$ by two-tailed Student's *t* test.

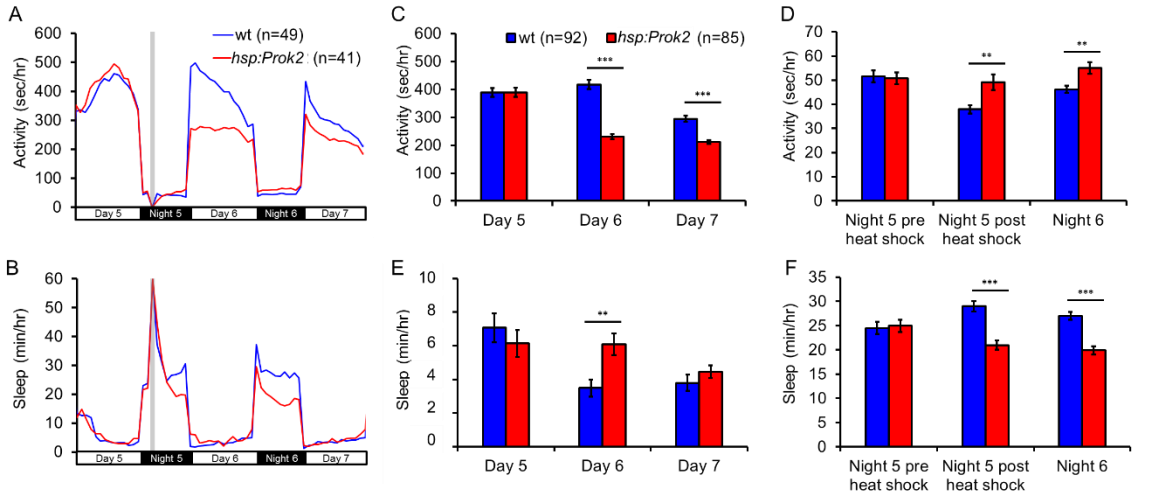

Figure 2.3. Prok2 overexpression promotes sleep during the day and activity at night following nighttime heat shock. Heat shock (grey bar) at night increases locomotor activity at night (A, D) and decreases locomotor activity during the day (A, C) for *Tg(hsp:Prok2)* larvae (red) compared to their non-transgenic siblings (blue). Prok2 overexpression also increases sleep during the day (B, E) and decreases sleep at night (B, F). These phenotypes were observed for up to 36 hours following heat shock. Data from one representative experiment (A, B) and two experiments combined (C-F) are shown. (C-F) show mean ± SEM. n indicates number of larvae analyzed. $*p < 0.05$, $***p < 0.001$ by two-tailed Student's *t* test. Note that (C-F) exclude the first two hours after heat shock to allow larvae to recover from the heat shock. Both genotypes slept much more during the recovery period.

Furthermore, Prok2-overexpressing larvae exhibited the opposite phenotype the following day (Figures 2.3A-2.3F), similar to the daytime phenotype observed following a daytime heat shock (Figure 2.2). These results indicate that Prok2 overexpression induces opposite sleep/wake phenotypes during the day and night.

2.6 Prok2 overexpression phenotype depends on lighting conditions and not circadian rhythms

The opposite sleep/wake phenotypes induced by Prok2 overexpression during the day and night might result from differential effects of the circadian clock or lighting

conditions on Prok2 function. To distinguish between these possibilities, we raised and tested larvae in either constant light or constant dark from birth, both of which result in larvae that lack overt molecular or behavioral circadian rhythms (Hurd and Cahill, 2002; Kaneko and Cahill, 2005; Prober et al., 2006; Gandhi et al., 2015). In constant light, Prok2 overexpression decreased locomotor activity for at least 36 hours and increased sleep for 12 hours (Figures 2.4A-2.4D). The lack of change in sleep after 12 hours could be due to a floor effect because both *Tg(hsp:Prok2)* larvae and their WT siblings slept very little in constant light conditions. We observed the opposite phenotype in constant dark; Prok2 overexpression increased locomotor activity for 24 hours and decreased sleep by ~50% for at least 36 hours (Figures 2.4E-2.4H).

To confirm the light dependency of Prok2 overexpression phenotype, we also raised larvae in normal light and dark condition but removed the external lighting cues after Prok2 overexpression. Larvae raised in normal light and dark condition continue to exhibit circadian rhythmicity of locomotor activity (Hurd et al., 1998; Hurd and Cahill, 2002). When given constant light after heat shock, Prok2 overexpression decreased locomotor activity for at least 2 days and increased sleep for 16 hours (Figures S2.2A-2.2F). We observed the opposite phenotype when given constant dark after heat shock; Prok2 overexpression increased locomotor activity and reduced sleep for at least 2 days (Figures S2.2G-2.2L). Together, these results suggest that the opposite effects of Prok2 overexpression on sleep/wake behaviors during the day and night are dependent on lighting conditions and not interactions with the circadian phase.

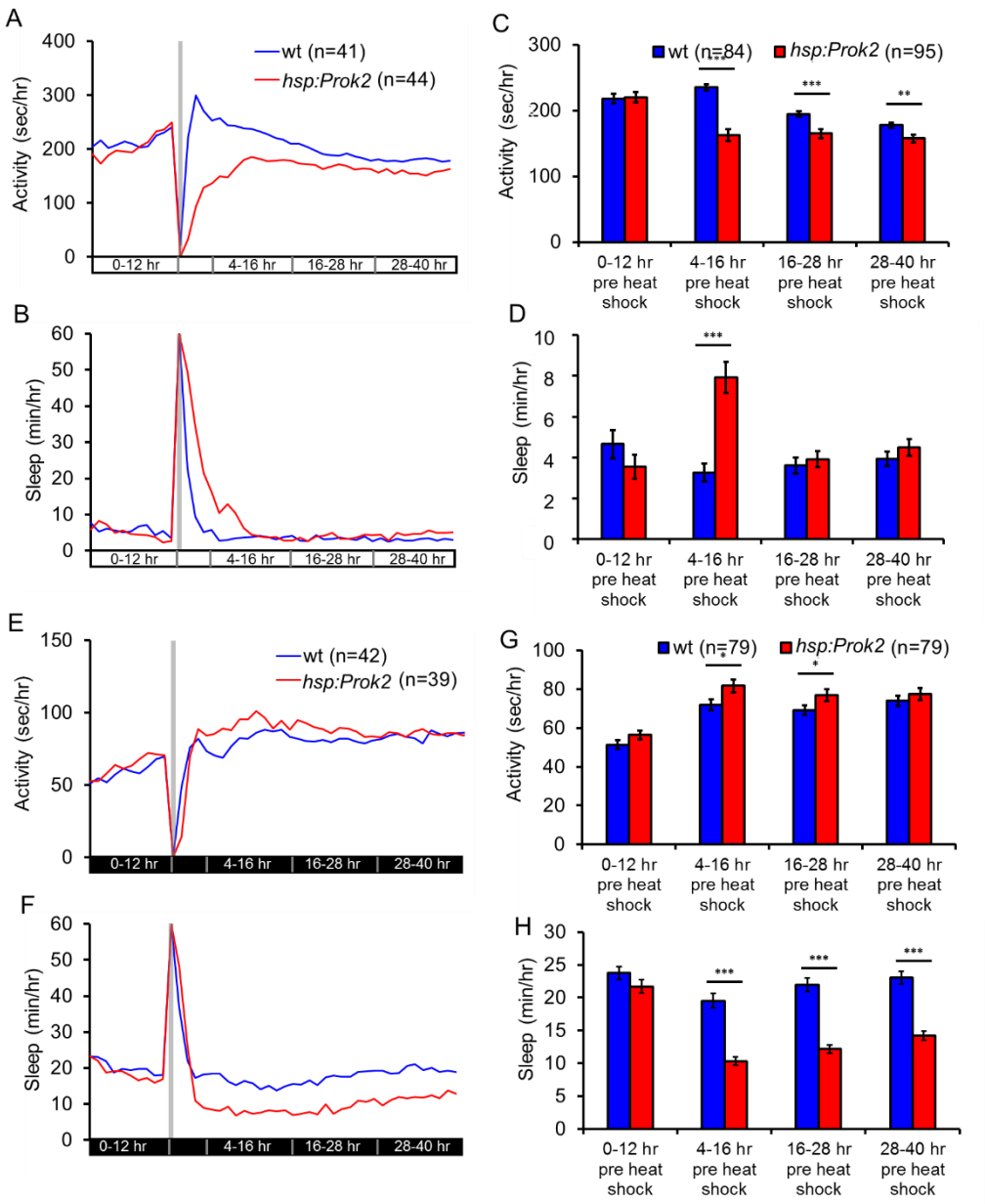

Figure 2.4. Prok2 overexpression phenotype depends on lighting condition and not on circadian rhythms. (A-D) Following heat shock at 5 dpf (grey bar), larvae raised and tested in constant light are less active for at least 36 hours (A, C) and sleep more for up to 16 hours (B, D). (E-F) Following heat shock at 5 dpf (grey bar), larvae raised and tested in constant dark are more active for up to 28 hours and sleep less for at least 36 hours. Data from one representative experiment (A, B, E, F) and two experiments combined (C, D, G, H) are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. *$p < 0.05$, ***$p < 0.001$ by two-tailed Student's *t* test. Note that (C-F) exclude the first two hours after heat shock to allow larvae to recover from the heat shock. *$p < 0.05$, **p<0.01, ***$p < 0.001$ by two-tailed Student's *t* test. Note that (C, D, G, H) exclude the first four hours after heat shock to allow larvae to recover from the heat shock.

2.7 *prok2* mutant zebrafish lack overt developmental defects

To examine the function of endogenous *prok2* in a diurnal animal, we generated zebrafish with a predicted null mutation in *prok2* (Figure 2.5A). *prok2* mutant mice exhibit a high rate of embryonic and neonatal lethality, and surviving animals are hypoactive and show defective olfactory, reproductive, and gonadotropin releasing hormone (GnRH) neuron development (Ng et al., 2005; Dod é et al., 2006; Pitteloud et al., 2007). We found that *prok2-/-* zebrafish are viable, fertile, and comparable in appearance to their *prok2*+/- and WT siblings (Figure S2.3A). Furthermore, a *prok2*+/- incross generated progeny of the expected Mendelian ratio (12/45 *prok2*+/+, 22/45 *prok2*+/-, and 11/45 *prok2*-/-). To investigate whether the zebrafish mutant exhibits developmental defects similar to the mouse mutant, we performed ISH using markers for the olfactory system (*tyrosine hydroxylase 1* (*th1*) and *empty spiracles homeobox 1* (*emx1*)), and the two zebrafish *gnrh* paralogs, *gnrh2* and *gnrh3.* In contrast to the mouse *prok2* mutant, we failed to detect abnormal expression of any of these markers, suggesting that the olfactory system and *gnrh*-expressing neurons develop normally in zebrafish *prok2-/-* mutant larvae. (Figure S2.3B). This result accords with our observation that *prok2* is only expressed in the larval zebrafish ventral hypothalamus (Figures 2.1A, 2.1B), whereas *prok2* is expressed in many other brain regions in rodents. These results suggest that the larval zebrafish *prok2* mutant may be more appropriate than the mouse mutant for studying the role of Prok2 signaling in sleep, since any mouse sleep phenotypes may be complicated by the presence of developmental defects.

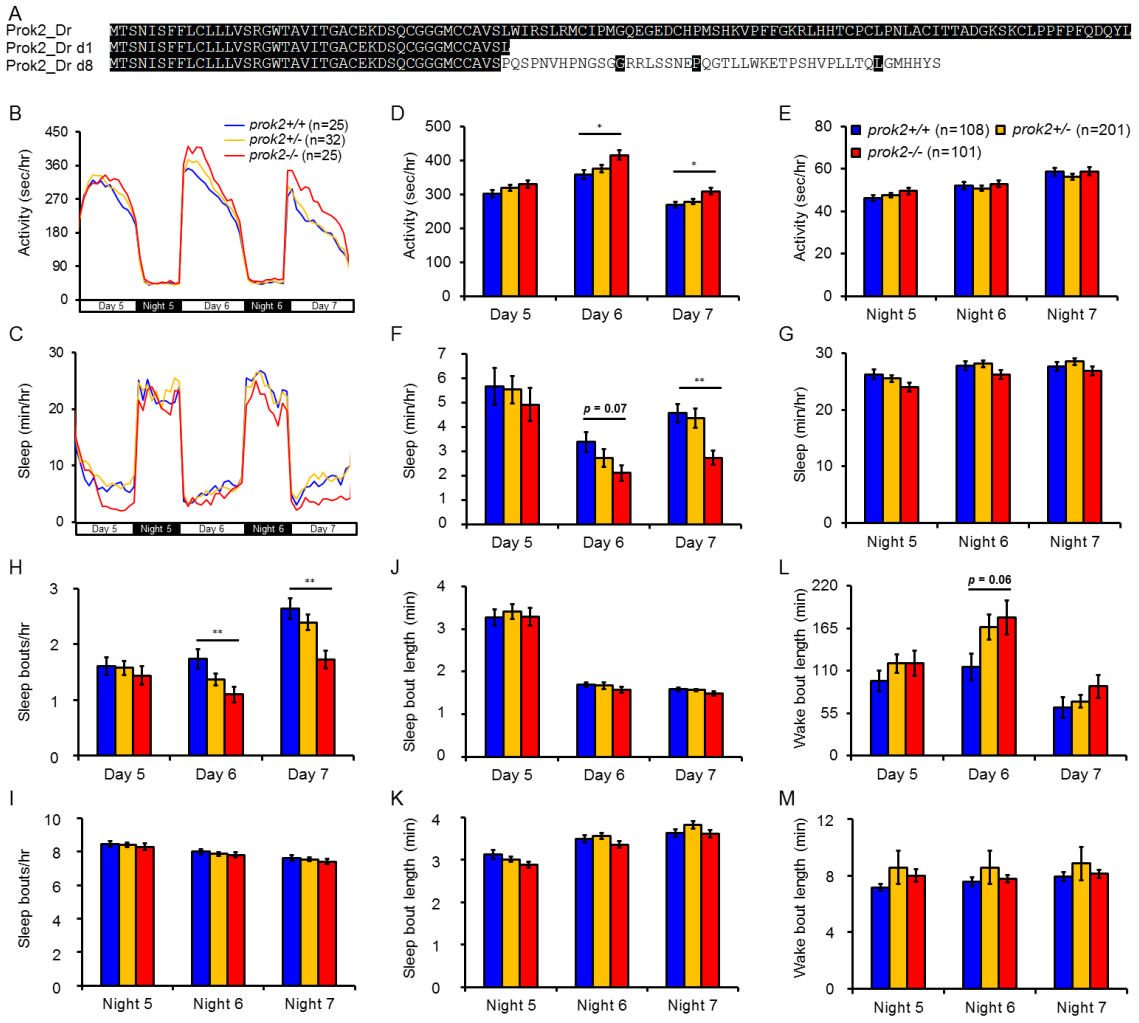

Figure 2.5. *prok2* mutant larvae are more active during the day. (A) The amino acid sequences of two zebrafish Prok2 mutant proteins (d1 and d8) generated in this study are shown compared to the WT protein. (B-M) During the day, *prok2*-/- larvae (red) are more active (B, D) and sleep less (C, F) compared to their *prok2*+/- (yellow) and *prok2*+/+ (blue) siblings. This phenotype results from a decrease in the number of sleep bouts (H), with a trend towards longer waking bouts (L). Note that this phenotype was observed at 6 dpf and 7 dpf, but not at 5 dpf. Data from one representative experiment (B, C) and five experiments combined (D-M) are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by one-way ANOVA and Dunnett's test using comparison to WT.

2.8 *prok2* mutant zebrafish larvae exhibit reduced sleep and hyperactivity during the day

To determine whether *prok2* is required for normal sleep/wake behaviors in larval zebrafish, we compared *prok2*-/- larvae to their *prok2*+/- and WT siblings (Figures 2.5B, 2.5C). Larvae of all three genotypes showed similar amounts of locomotor activity and sleep at night (Figures 2.5B, 2.5C, 2.5E, 2.5G). In contrast, *prok2*-/- larvae exhibited increased locomotor activity and decreased sleep compared to sibling controls during the day at 6 and 7 dpf (Figures 2.5B, 2.5C, 2.5D, 2.5F). The increase in activity and decrease in sleep during the day was primarily caused by a decreased number of sleep bouts (Figure 2.5H), with little or no effect on the length of sleep or wake bouts (Figures 2.5J, 2.5L). These results indicate that Prok2 is required to maintain normal daytime sleep/wake levels. Based on our gain- and loss-of-function results, we propose that Prok2 may act as a homeostatic regulator of sleep/wake states that opposes the predominant behavioral state during the day and at night (see discussion).

2.9 *prok2* mutant larvae do not exhibit attenuated sleep/wake rhythmicity

prok2 mutant mice are reported to have attenuated rhythmicity of several processes regulated by the circadian clock, including locomotor activity, core body temperature, and circulating glucose and cortisol levels (Li et al., 2006). To test whether *prok2* is required for the circadian regulation of locomotor activity and sleep in zebrafish larvae, we entrained *prok2*-/- and their sibling controls in 14:10 hour light:dark condition for 5 days and then monitored their behavior in free-running constant dark conditions (Figures 2.6A-2.6F). We found that the amount and timing of locomotor activity and

sleep was indistinguishable for all three genotypes, indicating that *prok2* is not required for the circadian regulation of locomotor activity or sleep in zebrafish larvae. This result is consistent with the observation that humans lacking *prok2* have normal circadian rhythmicity of body temperature and circulating cortisol and melatonin levels (Balasubramanian et al., 2014).

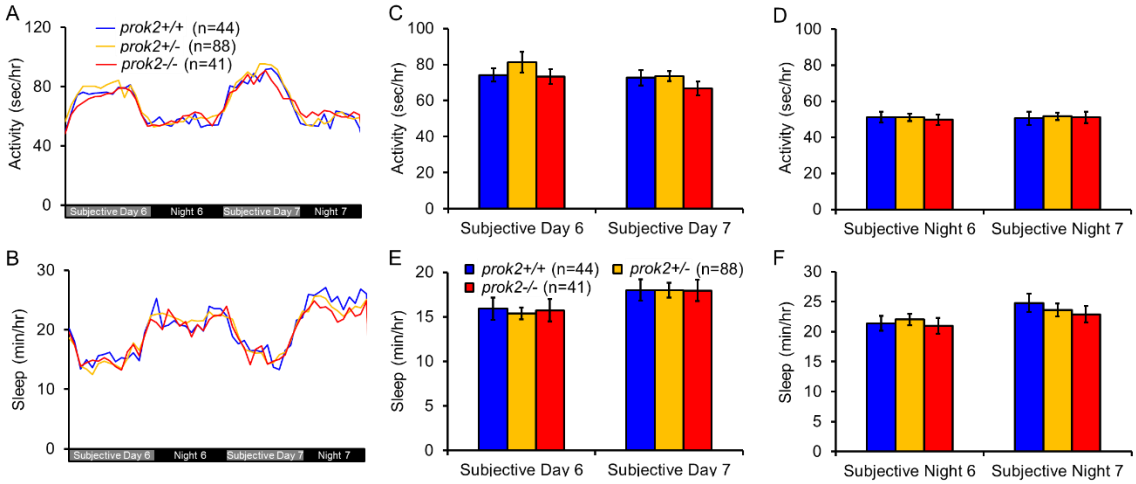

Figure 2.6. *prok2* mutant larvae exhibit normal sleep/wake rhythmicity. Larvae entrained in 14:10 hour light:dark conditions for 5 days and then monitored in constant dark maintained normal circadian rhythms of locomotor activity (A, C, D) and sleep (B, E, F), with no significant differences between *prok2*-/-, *prok2*+/-, or *prok2*+/+ siblings. Data from 2 experiments combined are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed.

2.10 Zebrafish Prok2 receptors are expressed in discrete brain regions

The mammalian genome contains two Prok2 receptors, Prokr1 and Prokr2, that have similar affinities for Prok2 *in vitro* (Lin et al., 2002). The zebrafish genome contains two Prokr paralogs, designated *prokr1a* (ENSDARG00000074182) and *prokr1l* (ENSDARG00000090315) (Figures S2.4A, S2.4B). Zebrafish Prokr1a protein is 62%

and 59% identical to mouse Prokr1 and Prokr2, respectively. Zebrafish Prokr1l protein is 66% and 69% identical to mouse Prokr1 and Prokr2, respectively.

To determine the expression patterns of these receptors in larval zebrafish, we performed ISH using animals fixed at 24 hours post-fertilization (hpf) and 5 dpf. We found that *prokr1* is expressed in a row of cells along the spinal cord and in a bilateral cluster of cells in the ventral hypothalamus (Figures 2.7A, 2.7A'). In contrast, *prokr2* is expressed in several discrete clusters of cells in the telencephalon, thalamus, hypothalamus, tuberomammillary nucleus, locus coeruleus, and medulla oblongata at 5 dpf (Figures 2.7B-2.7B'') In rodents, *prokr2* mRNA is widely detected throughout the brain, including in regions known to regulate sleep, such as the hypothalamus, periaqueductal grey, dorsal raphe, and mammillary nuclei (Masumoto et al., 2006). *prokr1* is expressed in a more restricted pattern in the mouse, including the olfactory region, dentate gyrus, zona incerta and dorsal motor vagal nucleus (Masumoto et al., 2006). Based on expression pattern similarities, we refer to zebrafish *prokr1a* as *prokr1* and *prokr1l* as *prokr2*.

To characterize *prokr2*-expressing cells, we performed double FISH using probes specific for *prokr2* and markers for neurotransmitter and neuromodulator cell types. We observed that *prokr2*-expressing cells in the telencephalon are GABAergic (Figures 2.7E-2.7E''), while a bilateral cluster in the thalamic region (Wullimann and Knipp, 2000) is glutamatergic (Figures 2.7C-2.7C''). All *prokr2*-expressing cells in the locus coeruleus and medulla oblongata express *vesicular monoamine transporter* (*vmat*)-EGFP (Figures 2.7D-2.7D'', 2.7F-2.7F''), and thus are noradrenergic (Wen et al., 2008). Approximately 30% of *prokr2*-expressing cells in the tuberomammillary nucleus express *histidine*

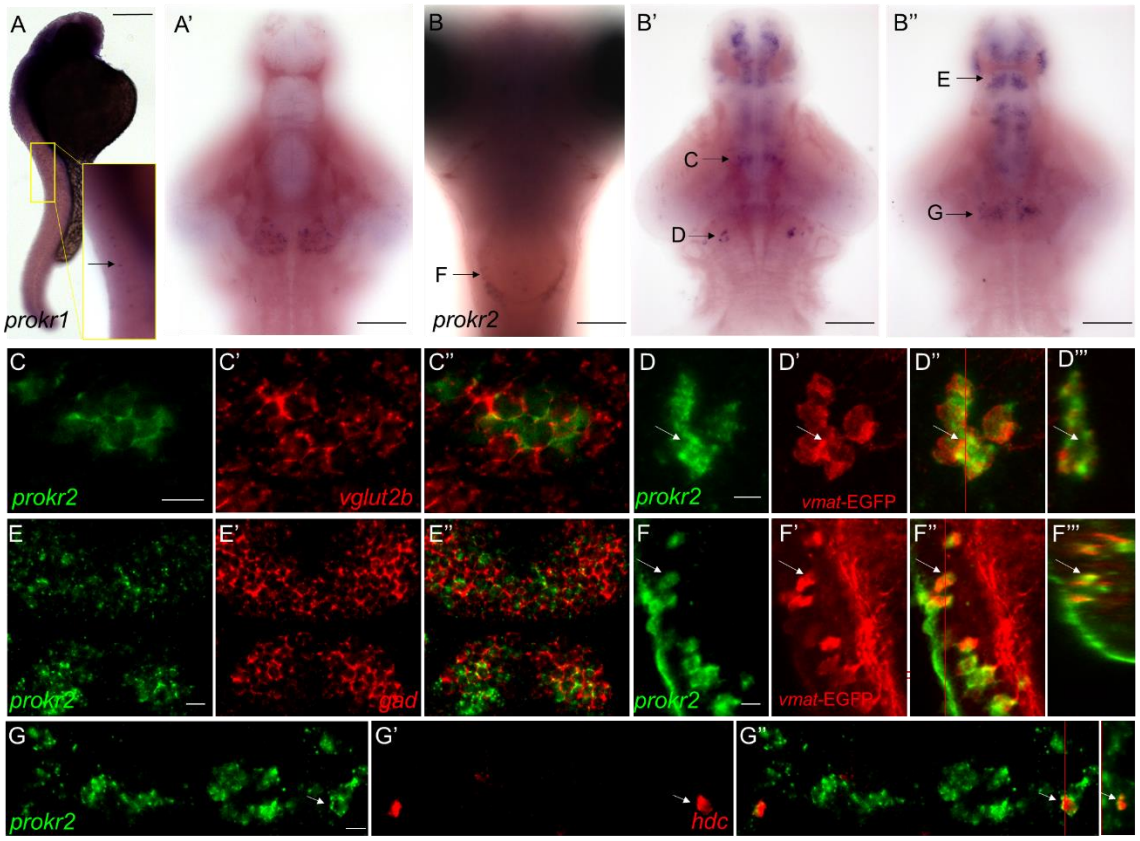

Figure 2.7. Expression patterns of *prokr1* and *prokr2* in larval zebrafish. *prokr1* is expressed in a row of neurons in the spinal cord at 24 hpf (A, enlarged in inset), and in a bilateral cluster of cells in the ventral hypothalamus at 5 dpf (A'). *prokr2* is expressed in discrete clusters of cells in the hindbrain (B), midbrain (B'), and forebrain (B'') at 5 dpf. Arrows indicate domains of *prokr2*-expressing cells that are shown at higher magnification in the indicated panels. Dorsal (B) and ventral (A', B', B") views are shown. (B') and (B") show dorsal and ventral optical sections, respectively. Rostral at top. 0.46 µm confocal sections show that *prokr2*-expressing neurons in the thalamic region expresses *vesicular glutamate transporter 2b* (*vglut2b*) (C) and *prokr2*-expressing neurons in the forebrain expresses *glutamate decarboxylase* (*gad65 and gad67* probes combined) (F). Confocal projections show that *prokr2*-expressing neurons in the locus coeruleus (D) and medulla oblongata (F) expresses *vesicular monoamine transporter* (*vmat*), which labels noradrenergic neurons in these brain regions. Approximately 30% of *prokr2*-expressing neurons in the tuberomammillary nucleus expresses *histidine decarboxylase* (*hdc*) (G), a marker of histamine neurons. C''', D''', E''', F''', and G''' show the orthogonal view of C'', D'', E'', F'', and G'' at the red line, respectively. Blue lines in C''' and E''' indicate the z-position in C'' and E'', respectively. Arrows in (C-G) indicate representative neurons. Scale bars: A-B, 100 µm; C-G, 10 µm.

decarboxylase (*hdc*), and thus are histaminergic (Figures 2.7G-2.7G''). Thus, *prokr2* is expressed in both GABAergic and glutamatergic cell types in the larval zebrafish brain, including in brain regions that are thought to promote arousal.

2.11 Prok2 overexpression-induced sleep/wake phenotypes require Prokr2 but not Prokr1

in vitro experiments have shown that mammalian Prok2 has similar binding affinity for both Prokr1 and Prokr2 (Lin et al., 2002); however, potential roles for these receptors in Prok2 signaling *in vivo* have not been tested. To determine whether these receptors are required for Prok2 signaling *in vivo*, we generated zebrafish with predicted null mutations in *prokr1* and *prokr2* (Figures S2.4A, S2.4B). Like the zebrafish *prok2* mutant, *prokr1* and *prokr2* single and double mutant zebrafish are homozygous viable and fertile and lack overt developmental defects (Figures S2.3A, S2.3B). We found that the Prok2 overexpression phenotype was abolished in *prokr2-/-* larvae (Figure 2.8A-2.8F), but was unaffected in *prokr1-/-* larvae (Figure S2.5A-2.5F). These results indicate that Prok2 overexpression-induced effects on sleep/wake behaviors require *prokr2* but not *prokr1*. In contrast to the zebrafish *prok2* mutant, *prokr1* and *prokr2* single and double mutants exhibited normal sleep/wake behaviors (Figures S2.4C-S2.4T), suggesting that there may be other endogenous ligands for these receptors.

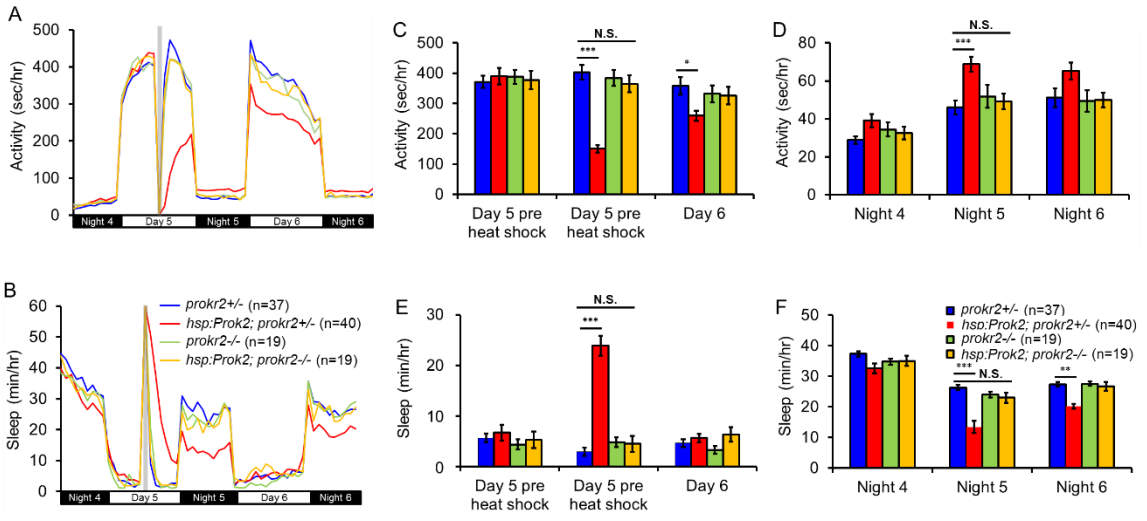

Figure 2.8. Prok2 overexpression phenotype requires Prokr2. Following heat shock (grey bar in A, B), Prok2 overexpression-induced locomotor activity (A, C, D) and sleep (B, E, F) phenotypes are abolished in *prokr2*-/- larvae (yellow), but not *prokr2*+/- larvae (red). Data from two experiments are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. **p*< 0.05, ***p* < 0.01, ****p* < 0.001 by two-way ANOVA and Tukey's multiple comparison test.

2.12 Prok2 overexpression phenotype persists in zebrafish containing mutations in other sleep regulatory pathways

In an attempt to identify the genetic mechanisms throughout which Prok2 signaling affects sleep/wake behaviors, we tested whether the Prok2 overexpression phenotypes are suppressed in larvae containing mutations in genes implicated in regulating sleep. Based on our observations that Prok2 overexpression-induced phenotypes require *prokr2* (Figure 2.8), and that *prokr2* is expressed in noradrenergic (NA) neurons in the locus coeruleus and medulla oblongata (Figures 2.7D-2.7D'', 2.7F-2.7F''), we hypothesized that Prok2-induced phenotypes might require NA. For example, Prok2 overexpression might promote daytime sleep by inhibiting NA signaling, and conversely might inhibit sleep at night by stimulating NA signaling. To test this

hypothesis, we overexpressed Prok2 in larvae containing a null mutation in *dopamine beta hydroxylase* (*dbh)*, which lack noradrenaline and exhibit reduced locomotor activity and increased sleep (Singh et al., submitted)(Figures S2.6A, S2.6B). During the day, Prok2 overexpression further reduced locomotor activity and increased sleep in *dbh*-/- larvae compared to their *dbh*+/- siblings (Figures S2.6A-S2.6C, S2.6E). At night, Prok2 overexpression significantly decreased sleep in *dbh*-/- larvae and showed a trend toward increased activity (Figures S2.6A, S2.6B, S2.6D, S2.6F). The lack of a different Prok2 overexpression phenotype for *dbh-/-* compared to *dbh*+/- larvae at 5 dpf may be due to a floor effect, as the magnitude of difference between *Tg(hsp:Prok2);dbh*-/- and *Tg(hsp:Prok2)*; *dbh*+/- larvae is comparable to that between *dbh*-/- and *dbh*+/- larvae on the following day. Based on these results, we conclude that Prok2 overexpression-induced sleep/wake phenotypes do not require NA.

We further tested whether the Prok2 overexpression phenotype is blocked in zebrafish containing mutations that affect other known sleep regulatory pathways or using small molecule regulators of these pathways (Table S1). We tested zebrafish with mutations in *hdc* (Chen and Prober, unpublished), *tryptophan hydroxylase 2 (tph2)* (Oikonomou and Prober, unpublished), *arylalkylamine N-acetyltransferase 2* (*aanat2*) (Gandhi et al., 2015) , *hypocretin receptor* (Yokogawa et al., 2007), or *glucocorticoid receptor* (Ziv et al., 2013), which lack histamine, serotonin in the dorsal raphe, melatonin, hypocretin signaling, and glucocorticoid signaling, respectively. We also tested the dopamine receptor 1 antagonist SCH23390 (Monti et al., 1990) and exogenous melatonin(Gandhi et al., 2015). None of these mutations or small molecules blocked the Prok2 overexpression phenotype (data not shown), suggesting that none of these

pathways is necessary for the effects of Prok2 overexpression on sleep/wake states. While we cannot rule out the possibility that Prok2-induced phenotypes act via more than one of these pathways, which must be blocked simultaneously to inhibit the effects of Prok2 overexpression, these results suggest that Prok2 affects sleep via other mechanisms.

2.13 Discussion

Prok2 has been proposed to act as a circadian output molecule that affects locomotor activity in nocturnal rodents (Cheng et al., 2002; Zhou and Cheng, 2005). However, it is unclear how the functions of Prok2 specifically, and circadian output factors in general, that were identified in nocturnal animals can be translated to diurnal animals. Here we describe the first diurnal vertebrate genetic gain- and loss-of-function models for Prok2 and use them to investigate the function of Prok2 in regulating diurnal sleep/wake behaviors. In nocturnal rodents, Prok2 expression oscillates in the SCN in a circadian manner with high levels during the day, and ICV infusion of Prok2 at night decreases locomotor activity (Cheng et al., 2002). Based on these observations, it was unclear whether Prok2 acts a behavioral signal that promotes inactivity or behaviors specific to the light phase (Cheng et al., 2002). We found that overexpressing Prok2 during the day in zebrafish larvae inhibits locomotor activity and promotes sleep, suggesting that Prok2 acts as a signal to promote inactivity and not for day-specific behaviors. In addition to inhibiting locomotor activity at night in nocturnal rodents, Prok2 infusion at night also resulted in increased locomotor activity the following day. While this phenotype was interpreted to be a consequence of reduced nighttime activity, we found that inducing Prok2 overexpression at night in larval zebrafish almost immediately

resulted in increased locomotor activity and decreased sleep. This observation suggests that Prok2 directly promotes both sleep during the day and activity at night. Surprisingly, we found that these opposite day/night behavioral phenotypes do not require circadian rhythms, but rather depend on lighting conditions, suggesting that Prok2 acts as a signal for light/dark cues, rather than day/night circadian cues.

Based on our observations, we propose a model in which Prok2 signaling acts as a homeostatic regulator of sleep/wake states that opposes the predominant behavioral state during light and dark periods. Thus, Prok2 overexpression in light, when diurnal animals are active and nocturnal animals are inactive, promotes inactivity in diurnal animals and activity in nocturnal animals. Similarly, Prok2 overexpression in dark, when diurnal animals are inactive and nocturnal animals are active, promotes activity in diurnal animals and inactivity in nocturnal animals. If this hypothesis is correct, diurnal *prok2* mutants should be more active during the day and less active at night. Indeed, we found that *prok2* mutant zebrafish larvae are more active and sleep less during the day. Similarly, *prok2* mutant nocturnal rodents also exhibit decreased sleep during the day (Li et al., 2006; Hu et al., 2007), suggesting that a role for endogenous Prok2 in regulating daytime sleep/wake behaviors may be conserved in diurnal and nocturnal vertebrates. However, inconsistent with our model, loss of *prok2* does not affect sleep/wake states at night in either zebrafish or rodents (Li et al., 2006; Hu et al., 2007). Thus, endogenous Prok2 may only regulate sleep/wake states during the day. Alternatively, because the zebrafish Prok2 overexpression phenotype at night is much weaker than the daytime phenotype, our behavioral assay may not be sensitive enough to detect a subtle nighttime *prok2* mutant phenotype. The lack of a nighttime phenotype in *prok2* mutants could also

result from redundant sleep-regulating mechanisms at night or developmental compensation for the loss of *prok2* that only rescues its nighttime function.

Previous studies showed that loss of Prok2 or Prokr2 in nocturnal rodents led to attenuated circadian rhythmicity, as measured by core body temperature, and circulating glucose and cortisol levels (Li et al., 2006; Prosser et al., 2007), as well as decreased daytime sleep. However, we found that circadian rhythmicity of locomotor activity and sleep was normal in larval zebrafish *prok2* mutants. This results suggests that Prok2 may not be required for the circadian regulation of sleep/wake behaviors in diurnal animals, consistent with the finding that melatonin is essential for the circadian regulation of sleep in zebrafish larvae (Gandhi et al., 2015). Alternatively, Prok2 function may differ in diurnal zebrafish and diurnal mammals. Further studies are required to determine whether other behavioral and physiological processes that are regulated by the circadian clock are compromised in zebrafish *prok2* mutants.

A potentially important difference between larval zebrafish and both nocturnal and diurnal rodents is that *prok2* expression oscillates in a circadian manner in the rodent SCN (Cheng et al., 2002), but we failed to detect any significant change in larval zebrafish *prok2* expression throughout the circadian cycle. It is unknown whether *prok2* expression oscillates in the human SCN. Thus, it is unclear whether circadian oscillation of *prok2* expression is unique to rodents, or whether *prok2* expression is differentially regulated in mammals and zebrafish. The latter possibility is consistent with the observation that the circadian clock of each larval zebrafish cell can be directly entrained by light (Whitmore et al., 2000) due to the presence of D and E boxes in the promoter of *period2* gene (Vatine et al., 2009). Because zebrafish larvae are optically transparent, it

may not be necessary for *prok2* expression to be regulated by the circadian clock, and it is unknown whether zebrafish larvae possess a master circadian pacemaker analogous to the mammalian SCN. Consistent with this hypothesis, *prok2* expression may oscillate in adult zebrafish (Noonin et al., 2013), which are no longer transparent, although the reported oscillation was not very robust. Furthermore, *prok2* is apparently expressed in several brain regions in adult zebrafish and it is unknown where this oscillation may occur. Analysis of *prok2* expression in additional species is needed to address this question.

Another distinction between rodents and zebrafish larvae is that *prok2* is expressed in many brain regions in addition to the SCN in rodents, including the nucleus accumbens, globus pallidus, medial amygdala, medial preoptic area, arcuate nucleus, and olfactory bulb (Cheng et al., 2002, 2006), which are involved in many biological processes. Accordingly, *prok2* mutant rodents suffer from embryonic and neonatal lethality as well as defects in the olfactory and reproductive systems. These defects may account for the reduced rhythmicity of processes regulated by the circadian clock in *prok2* mutant rodents. In contrast, *prok2* is exclusively expressed in the larval zebrafish ventral hypothalamus near the optic chiasm, a brain region similar to the mammalian SCN. This restricted expression pattern likely explains the lack of developmental defects in zebrafish *prok2* mutants, and may also account for the attenuated locomotor rhythms observed in the mouse mutant, but not the zebrafish mutant, in free-running conditions. Larval zebrafish may thus provide a simpler system to study the role of Prok2 in regulating sleep and circadian processes due to its restricted expression domain. The zebrafish *prok2* mutant phenotype also bears similarities to humans, as patients with

prok2 mutations exhibit normal circadian rhythmicity of melatonin, cortisol and core body temperature (Balasubramanian et al., 2014), but exhibit sleep defects (Dodé et al., 2006; Cole et al., 2008). These data agree with our observation that *prok2* mutant larval zebrafish exhibit abnormal sleep/wake behaviors but lack changes in free-running circadian rhythmicity, suggesting that larval zebrafish, being a diurnal vertebrate, may be a more relevant model than nocturnal rodents for studying Prok2 regulation of sleep in humans.

Previous studies identified two GPCRs, Prokr1 and Prokr2, that bind Prok2 with similar affinity *in vitro* (Lin et al., 2002). Prokr2 was suggested to be the endogenous receptor for Prok2 based on its expression in SCN target regions in rodents (Cheng et al., 2002), but this hypothesis has not been tested. We found that Prok2 overexpression phenotypes are abolished in *prokr2*, but not *prokr1*, mutant larvae, consistent with the hypothesis that Prokr2 mediates the effects of Prok2 on sleep/wake behaviors *in vivo*. Despite the sleep phenotype observed in *prok2* mutant zebrafish larvae, we failed to detect sleep defects in either single or double *prokr1* and *prokr2* mutants. This discrepancy suggests that there may be additional receptors for Prok2 that are not required for Prok2 overexpression phenotypes but are sufficient to maintain normal sleep/wake behaviors in the absence of *prokr1* and *prokr2*. Alternatively, there may be other ligands for these receptors that have opposite effects on sleep as Prok2.

In larval zebrafish, *prokr2* is expressed in several known sleep/wake centers, including locus coeruleus noradrenergic (NA) neurons and tuberomammillary nucleus histaminergic (HA) neurons. Prokr2 is not expressed in these regions in the rodent brain, suggesting that Prok2 signaling may act via different neuronal mechanisms in nocturnal

and diurnal animals. Alternatively, the simpler brain structure of zebrafish larvae or their diurnal nature may account for these differences. For example, nocturnal rodents may require an intermediate step between Prok2 output from the SCN to these sleep regulating centers. For example, in rodents, Prokr2 is expressed in the dorsomedial hypothalamic nucleus and amygdala, both of which innervate the locus coeruleus (Aston-Jones et al., 2001; Bouret et al., 2003; Zhou and Cheng, 2005; Cheng et al., 2006).

Even though *prokr2* is expressed in NA and HA neurons in larval zebrafish, the Prok2 overexpression phenotype persisted in mutants that lack NA or HA, suggesting that Prok2 does not affect sleep via these neuromodulators. The Prok2 overexpression phenotype was also unaffected in larvae that lack serotonin in the dorsal raphe, hypocretin signaling or glucocorticoid signaling, or in larvae treated with a dopamine receptor 1 antagonist. It remains possible that it is necessary to block more than one of these pathways simultaneously to inhibit the effects of Prok2 overexpression on sleep. Alternatively, Prok2 may act via known sleep regulatory pathways that we did not test or via unknown sleep regulators. The opposite phenotypes of Prok2 overexpression in light and dark suggests that factors that are differentially regulated by lighting conditions play a key role in determining the effects of Prok2 signaling on sleep. A good candidate factor is melatonin, whose synthesis is restricted to the night and is rapidly inhibited by light (Klein, 2007), but we found that neither depletion of endogenous melatonin nor addition of exogenous melatonin blocked the Prok2 overexpression phenotypes. It may nevertheless be useful to identify additional such factors and determine whether they modulate the effects of Prok2 signaling.

In summary, we have shown how a circadian clock output factor identified in nocturnal rodents affects sleep/wake behaviors in a diurnal vertebrate. Our results are consistent with observations in humans containing *prok2* mutations, suggesting that larval zebrafish may be more appropriate than nocturnal rodents as a model to understand how Prok2 regulates sleep in humans. It will be useful in the future to determine whether our findings are broadly applicable to other such factors, and thus reveal general principles for how observations in nocturnal animals may be translated to diurnal animals.

2.14 Materials and Methods

Ethics statement

Zebrafish experiments and husbandry followed standard protocols (Westerfield, 1993) in accordance with Caltech Institutional Animal Care and Use Committee guidelines.

Transgenic and mutant zebrafish

Heat-shock promoter regulated Prok2 (Tg(hsp:Prok2)). Full-length zebrafish *prok2* cDNA was cloned downstream of the zebrafish *hsp70c* promoter (Halloran et al., 2000) in a vector containing flanking I-SceI endonuclease recognition sites. Stable transgenic fish were generated by injecting plasmids with I-SceI into zebrafish embryos at the one-cell stage. Transgenic founders were identified by outcrossing potential founders, heat shocking progeny at 5 dpf, fixing larvae 30 minutes after heat shock and performing ISH using a *prok2*-specific probe.

Zebrafish mutants were generated using the TAL effector nuclease (TALEN) method as described (Reyon et al., 2012) using plasmids obtained from Addgene.

prok2 mutant. TALEN target sites were 5'-TGGCATGTGTTGTGCAGT-3' and 5'-TGCACATTCGGAGACT-3'. Two mutants were isolated and tested. Mutant d1 contains a 1 bp deletion (nucleotide 127 of the open reading frame: 5'-G-3'), which results in a change in reading frame after amino acid 41 and a premature stop codon after amino acid 42 compared to 107 amino acids for the WT protein. Mutant d8 contains an 8 bp deletion (nucleotides 124-131 of the open reading frame: 5'- TGTGGATC -3'), which results in a change in reading frame after amino acid 41 and a premature stop codon after amino acid 87 compared to 107 amino acids for the WT protein. The predicted mutant protein lacks key functional domains including 6 out 10 cysteine residue and is likely non-functional (Bullock et al., 2004). This assertion is supported by the observation that a mutant form of *prok2* identified in humans with Kallmann syndrome that was shown to be inactive *in vitro* was truncated at amino acid 54 (Pitteloud et al., 2007). The two zebrafish *prok2* mutants exhibited similar phenotypes and mutant d8 was used for all reported experiments. *prok2* mutants were genotyped using the primers 5'CAAGTGGACACACCGAACAC-3' and 5'-ATCCTGGAATGGAAATGGTG-3'. PCR products were then digested with BamHI (R0136, NEB). Mutant (404 bp) and WT (155 bp + 257 bp) bands were distinguished by running the digested PCR product on a 2% agarose gel.

prokr1 mutant. TALEN target sites were 5'-TACTTGAGGACTGTGTC-3' and 5'-TGGCCAGCAGAGCATT-3'. Two mutants were isolated and tested. Mutant d7 contains a 7 bp deletion (nucleotides 411-422 of the open reading frame: 5'-TACGTGTCCAC-3' was replaced by 5'-TTGGT3'), which results in a change in reading frame after amino acid 136 and a premature stop codon after amino acid 156 compared to 385 amino acids for the WT protein. Mutant d22 contains a 22 bp deletion (nucleotides 405-426 of the open reading frame: 5'- TCTCTCTACGTGTCCACCAATG-3'), which results in a change in reading frame after amino acid 134 and a premature stop codon after amino acid 151 compared to 385 amino acids for the WT protein. Both predicted mutant proteins lack more than 3 transmembrane domains from the C terminus and are therefore likely non-functional (Lin et al., 2002). The mutants exhibited similar phenotypes and mutant d7 was used for all reported experiments. *prokr1* mutants were genotyped using the primers 5'-GGCGTTGGTAATTGCGTATT-3' and 5'-TATCAGCCACCAGCACTCTG-3'. PCR products were then digested with AflIII (R0541, NEB). Mutant (527 bp) and WT (218 bp + 316 bp) bands were distinguished by running the digested PCR reaction on a 2% agarose gel.

prokr2 mutant. TALEN target sites were 5'-TCTCACAGAAACAGCCAT-3' and 5'-TACAGCTGCCACGTGGCT-3'. Two mutants were isolated and tested. Mutant d1 contains a 1 bp deletion (nucleotide 12 of the open reading frame: 5'- C-3'), which results in a change in reading frame after amino acid 4 and a premature stop codon after amino acid 13 compared to 396 amino acids for the WT protein. Mutant d14 contains a 14 bp deletion (nucleotides 7-20 of the open reading frame: 5'- GACGCCAATATCAG -3'),

which results in a change in reading frame after amino acid 2 and a premature stop codon after amino acid 21 compared to 396 amino acids for the WT protein. The predicted mutant protein lacks all 7 transmembrane domains and is likely non-functional (Lin et al., 2002). The mutants exhibited similar phenotypes and mutant d1 was used for all reported experiments. *prokr2* mutants were genotyped using the primers 5'-TGAGCGTAATGCTAATGGTCT-3' and 5'-CCAGAGTGGCGATAAACACA-3'. PCR products were then digested with BsaHI (R0556, NEB). Mutant (428 bp) and WT (190 bp + 239 bp) bands were distinguished by running the digested PCR reaction on a 2% agarose gel.

The *ET(vmat2:EGFP)* line has previously been described (Wen et al., 2008).

Behavioral experiments. Videotracker experiments were performed as previously described (Prober et al., 2006; Rihel et al., 2010b; Gandhi et al., 2015)). Larval zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5ºC with lights on at 9 am and off at 11 pm, except for constant light and dark experiments where larvae were raised in constant light or dark conditions at 28.5ºC. On the fourth day of development, individual larvae were placed in each wells of a 96-well plate (7710-1651, Whatman) containing 650 µL of E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.4). Plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. Locomotor activity was monitored using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch Monochrome camera (Dragonfly 2, Point Grey) fitted with a variable-focus megapixel

lens (M5018-MP, Computar) and infrared filter. The movement of each larva was recorded using the quantization mode. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and with customizable white light. The 96-well plate was housed in a chamber filled with recirculating water to maintain a constant temperature of 28.5 ℃. The parameters used for detection were: detection threshold, 15; burst, 29; freeze, 3; bin size, 60 seconds. Heat shocks were performed by removing the 96-well plate from the videotracker and placing it in a 37ºC water bath for 1 hour. At the end of each experiment, each well was examined and those containing bubbles (introduced during the sealing process) or more or less than a single larva were excluded from analysis. Videotracker sleep/wake data was processed using custom PERL and Matlab (version R2013a, The Mathworks, Inc) scripts. Any one minute period with less than 0.1 second of total movement was defined as one minute of sleep (Prober et al., 2006). A sleep bout was defined as a continuous string of sleep minutes. Average activity was defined as the average amount of detected activity in seconds/hour, including all rest bouts. Statistical tests were performed using Prism 6 (GraphPad).

***in situ* hybridization (ISH) and immunohistochemistry.** Samples were fixed in 4% paraformaldehyde for 16 hours at room temperature. ISH was performed using digoxigenin (DIG)-labeled antisense riboprobes (Thisse and Thisse, 2008). Antisense *prok2* riboprobe for was generated from a zebrafish EST (Genbank EB931495; Open Biosystems, Inc.). Antisense *prokr1* riboprobe was generated using a PCR fragment as template. The fragment was amplified using primers 5’-

TGACTCGCAGTCACACAGTTC-3' and T7 promoter-coupled 5'-GAATTGTAATACGACTCACTATAGGGTCTTCCAAAGTATGGGTCGAA-3'.

Antisense *prokr2* riboprobe was generated using a PCR fragment as template. The fragment was amplified using primers 5'-GCACAGAGAATGAGCGTCTG-3' and T7 promoter-coupled 5'-GAATTGTAATACGACTCACTATAGGGTCACTGAGGCTGAGGGTATAAA-3'.

Images were acquired using a Zeiss Axio ImagerM1 microscope. Fluorescent ISH used DIG-, 2, 4-dinitrophenol (DNP)- and fluorescein-labeled antisense riboprobes with the TSA Plus DNP System (PerkinElmer). For immunohistochemistry, rabbit polyclonal anti-GFP (1:1000; MBL International, #598) and Alexa Fluor 568 goat anti-rabbit (1:500; Invitrogen, #A-11011) antibodies were used. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (268298, EMD Millipore) staining was performed in 1:5000 in 2% DMSO/PBS + 0.25% triton X-100 for at least 4 hours at room temperature. Samples were mounted in 50% glycerol/PBS, and imaged with a Zeiss LSM 780 laser-scanning confocal microscope with 405 nm, 488 nm, and 561 nm lasers and 40x objective. Images were processed using Fiji (Schindelin et al., 2012).

Quantitative reverse transcription-PCR. Larval zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5^{o}C with lights on from 9 am to 11 pm. Total RNA was isolated using RNeasy (74106, Qiagen) from 25 pooled larvae at 11am, 4 pm, 9 pm, 1 am, 4 am, and 8 am at 6 dpf , and at 11 am at 7 dpf. cDNA was synthesized from 5 µg of total RNA using Superscript III Reverse Transcriptase (18080-051, Invitrogen) and quantitative PCR was carried out using SYBR green master mix (4364346, Life

Technologies) in triplicate on an ABI PRISM 7900HF (Life Technologies) instrument. ΔCt was calculated using *actin* as a reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method by normalizing to the sample with the highest ΔCt value for each gene (Schmittgen and Livak, 2008). Primers for amplification: *prok2*, 5'-GGGGCATGTGAGAAGGACTCT-3' and 5'-TCTTCTCCCTCCTGACCCATT-3'; *per3*, 5'-CTCCAGCTTTCACAGCACTCA-3' and 5'-ACGCTTCTTCATCTCCTGCAC-3'; *actin*, 5'-TCCTCCCTGGAGAAGAGCTATG-3' and 5'-TCCATACCCAGGAAGGAAGG-3'.

***prok2* fluorescent *in situ* hybridization.** Larvae were raised in on a 14 hour:10 hour light:dark cycle at 28.5C with lights on from 9 am to 11 pm until 6 dpf. Samples were fixed in 4% PFA overnight at room temperature and fluorescent *in situ* hybridization was performed on dissected brains using a DIG-labeled antisense riboprobe specific for *prok2* and the TSA Plus System (NEL741001, PerkinElmer). Samples were developed using Cy3 amplification reagent at 1:200 for 10 minutes. Imaging was performed using Zeiss 780 confocal microscope using the same settings for all samples (40x objective). Total integrated pixel density was measured by subtracting background integrated pixel density from total integrated pixel intensity using ImageJ.

Supplemental figures and table

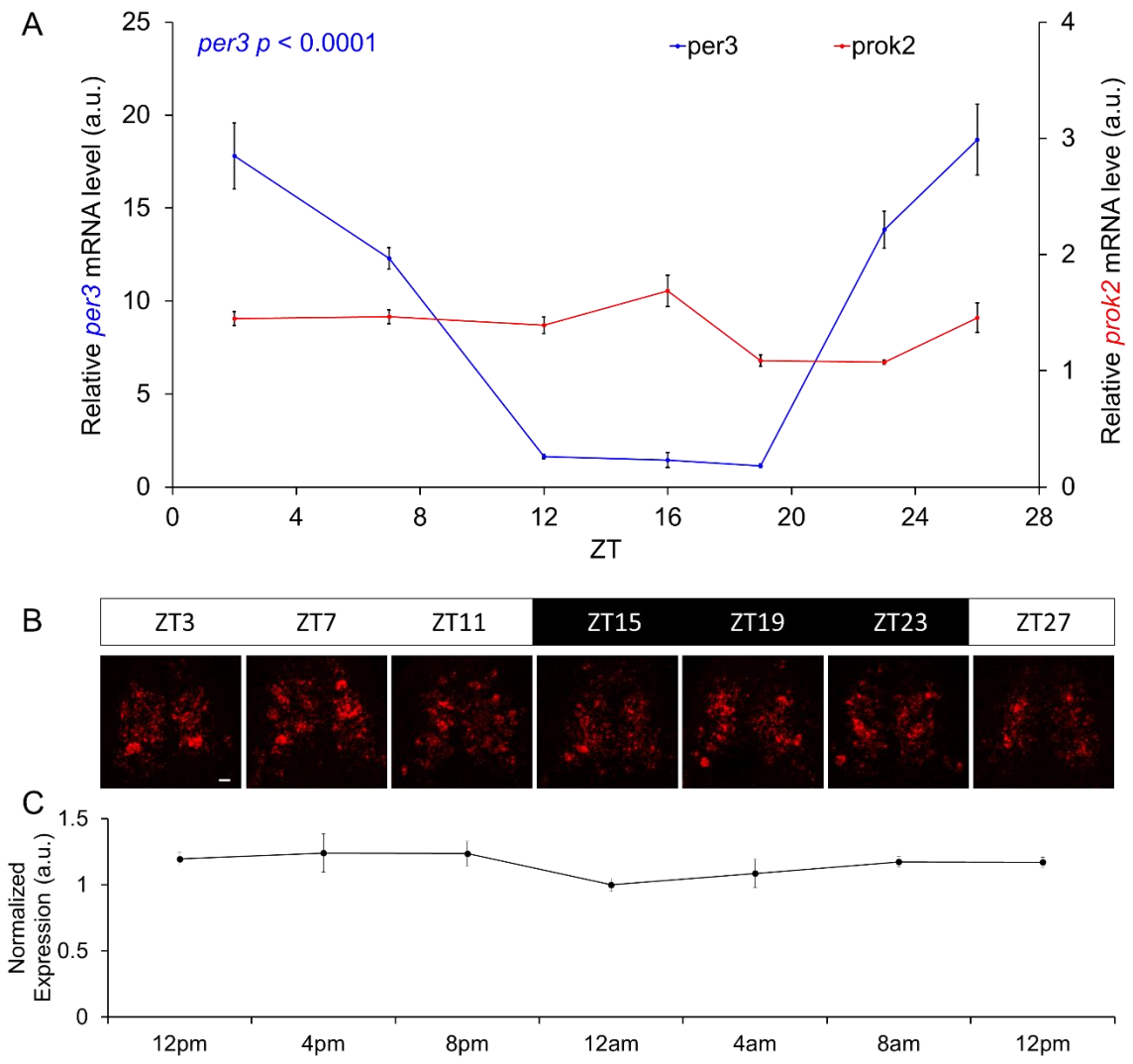

Figure S2.1. *prok2* expression does not oscillate in circadian manner in larval zebrafish. (A) qRT-PCR analysis of *prok2* and *per3* mRNA are shown. *prok2* expression does not change significantly over 24 hours, while *per3* expression oscillates with a 24 hour period, compared with actin. Larvae were raised in 14:10 hr light:dark conditions and collected at the indicated times beginning at 2 am (ZT 2) at 6 dpf. Triplicate biological samples were collected at each time point. Mean ± SEM values are shown (***$p < 0.001$ by one-way ANOVA) followed by Tukey's test). (B) Representative images of *prok2* FISH in larval zebrafish brains fixed at the indicated time. *prok2* mRNA levels appear to be similar at all circadian time points. Scale bar: 10 μm. (C) Quantification of total *prok2* fluorescence pixel intensity is shown. *prok2* expression does not oscillate (peak:trough ratio=1.24, p=0.40 by one-way ANOVA). Mean ± SEM is shown. Five brains were quantified for each time point.

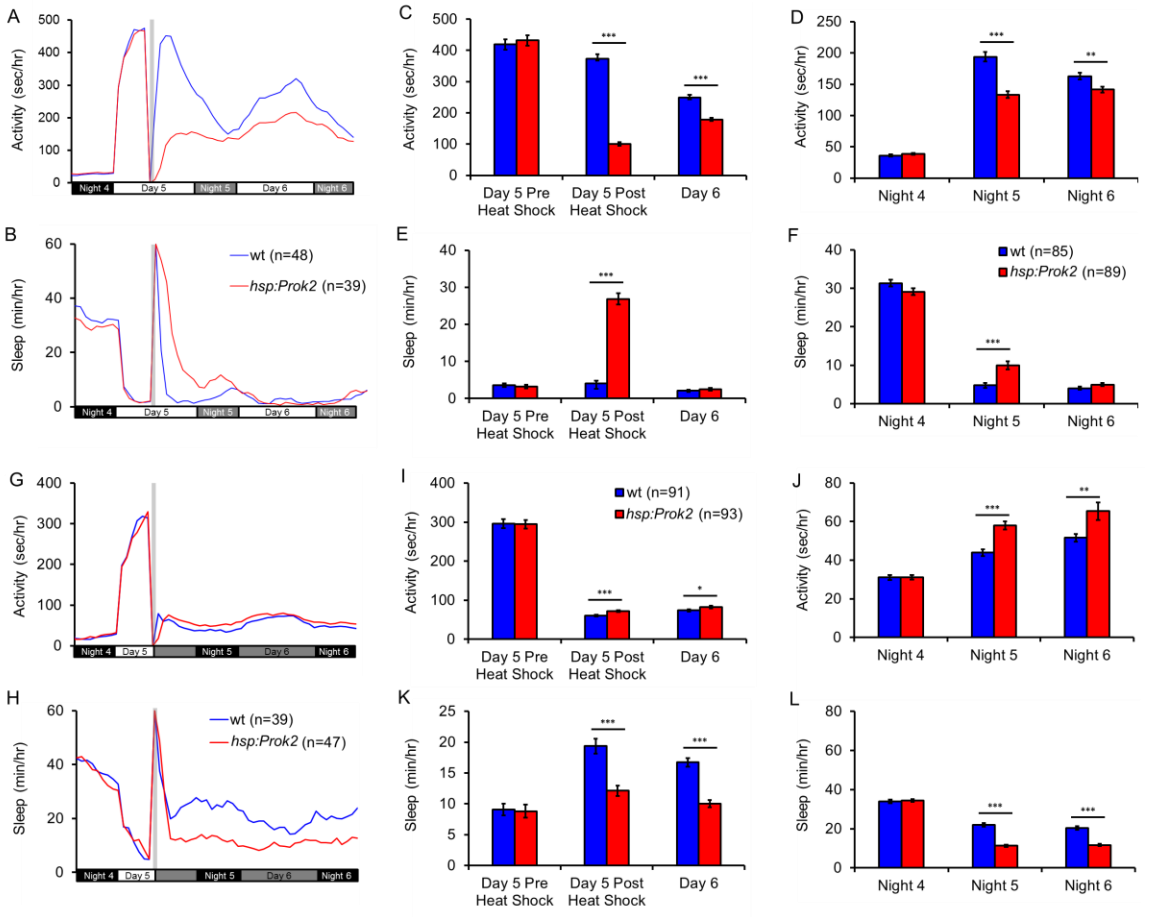

Figure S2.2. The Prok2 overexpression phenotype depends on lighting condition and not on circadian rhythms. (A-F) Following heat shock at 5 dpf (grey bar), larvae raised in normal light dark cycle and tested in constant light are less active for at least 2 days (A, C) and sleep more for up to 16 hours (B, D). (G-L) Following heat shock at 5 dpf (grey bar), larvae raised and tested in constant dark are more active and sleep less for at least 2 days. Data from one representative experiment (A, B, G, H) and two experiments combined (C-F, I-L) are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. *$p < 0.05$, ***$p < 0.001$ by two-tailed Student's *t* test. Note that (I, K) exclude the first two hours after heat shock to allow larvae to recover from the heat shock. *$p < 0.05$, **p<0.01, ***$p < 0.001$ by two-tailed Student's *t* test.

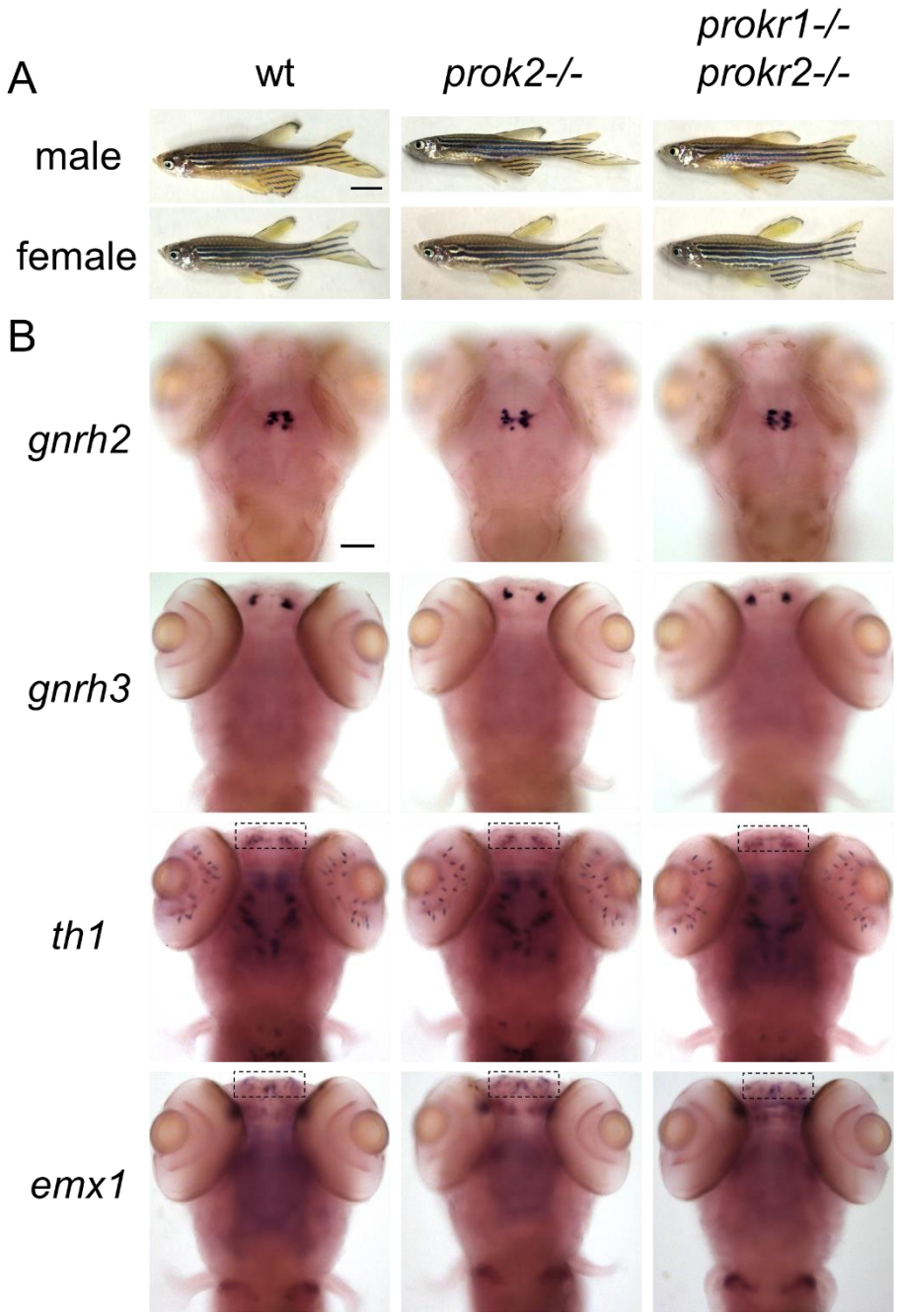

Figure S2.3. *prok2* and prok receptor mutants do not show apparent development defects. (A) Images of representative WT, *prok2-/-*, and *prokr1-/-; prokr2-/-* adult zebrafish are shown. All fish are similar in size and morphology. (B) Representative images of ISH using probes specific for *gonadotropin-releasing hormone 2* (*gnrh2*), *gnrh3*, *tyrosine hydroxylase 1* (*th1*) and *empty spiracles homeobox 1* (*emx1*) in WT, *prok2-/-*, and *prokr1-/-*; *prokr2-/-* larvae are shown. Dashed boxes indicate olfactory region. Larvae of all genotypes show similar expression levels and patterns of each gene. Scale bars: (A) 5 mm. (B) 100 μm.

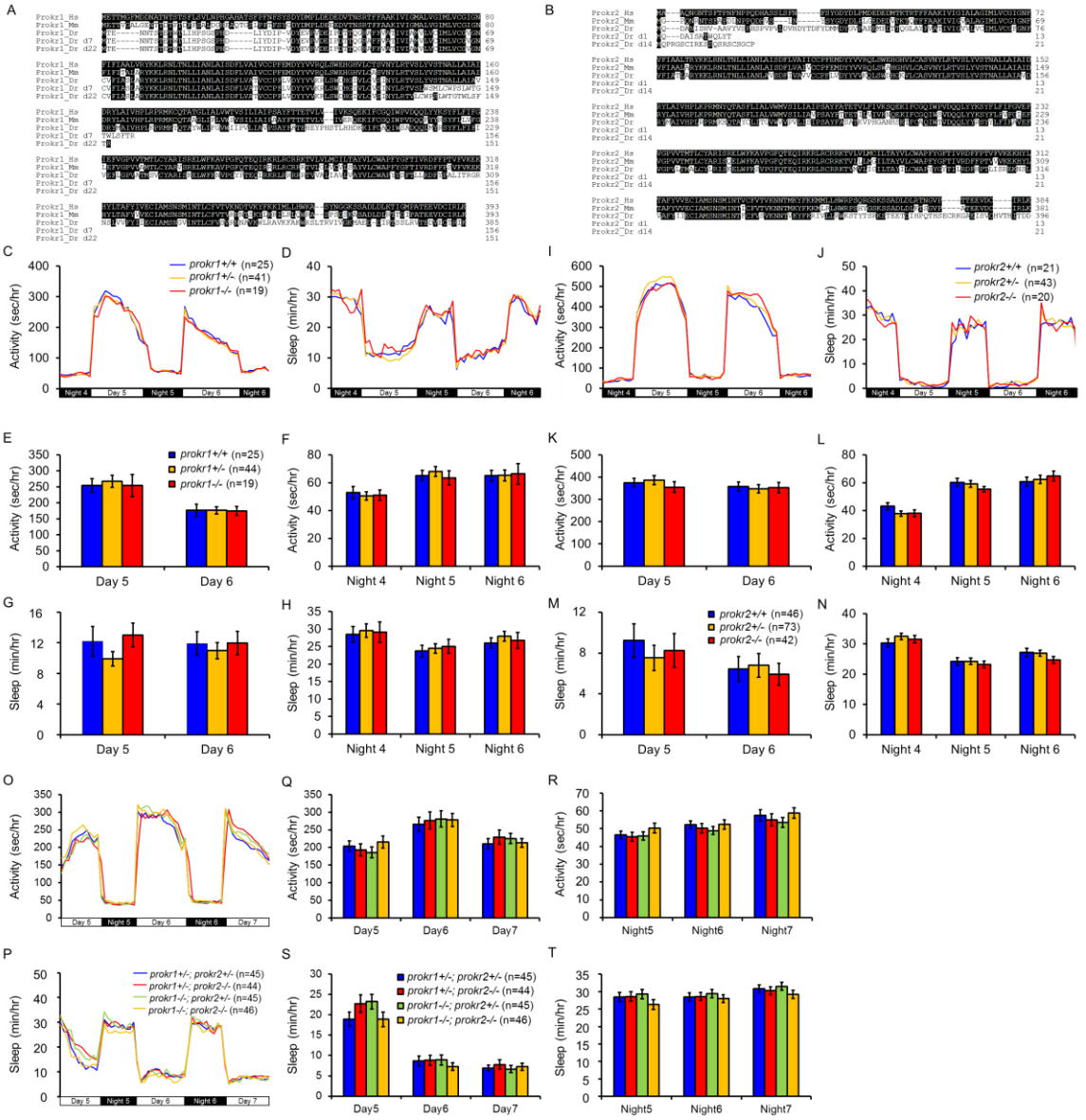

Figure S2.4. *prok2* receptor mutants lack sleep/wake phenotypes. (A, B) Amino acid sequence alignments of human (Hs), mouse (Mm), and zebrafish (Dr) Prokr1 (A) and Prokr2 (B) orthologues, and the sequences of two zebrafish Prokr1 mutant proteins (d7 and d22) and two zebrafish Prokr2 mutant proteins (d1 and d14) that were generated in this study are shown. Prokr1 mutant proteins are truncated at the 4th transmembrane domain. Prokr2 mutant proteins are truncated before the 1st transmembrane domain. (C-H) *prokr1-/-*, *prokr1+/-*, and *prokr1+/+* larvae showed similar amounts of locomotor activity (C, E, F) and sleep (D, G, H) during the day and night. (I-N) *prokr2-/-*, *prokr2+/-*, and *prokr2+/+* larvae showed similar amounts of locomotor activity (I, K, L) and sleep (J, M, N) during the day and night. (O-T) *prokr1+/-; prokr2+/-*, *prokr1-/-; prokr2+/-*, *prokr1+/-; prokr2-/-*, and *prokr1-/-; prokr2-/-* larvae showed similar amounts of locomotor activity (O, Q, R) and sleep (P, S, T) during the day and night. Data from one experiment is shown for Prokr1 mutant. Data from two experiments are shown for Prokr2

mutant and Prok double receptor mutants. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by one-way ANOVA and Dunnett's test using comparison to WT.

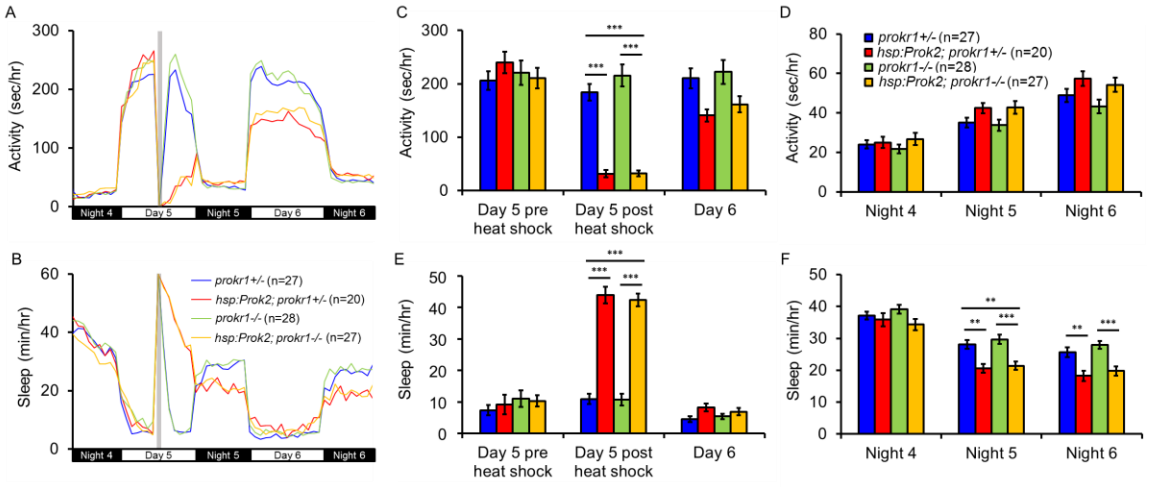

Figure S2.5. Prok2 overexpression phenotype does not require Prokr1. Following heat shock (grey bar in A, B), Prok2 overexpression-induced locomotor activity (A, C, D) and sleep (B, E, F) phenotypes were similar in *prokr1*-/- (yellow) and *prokr1*+/- larvae (red). Data from two experiments are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA and Tukey's multiple comparison test.

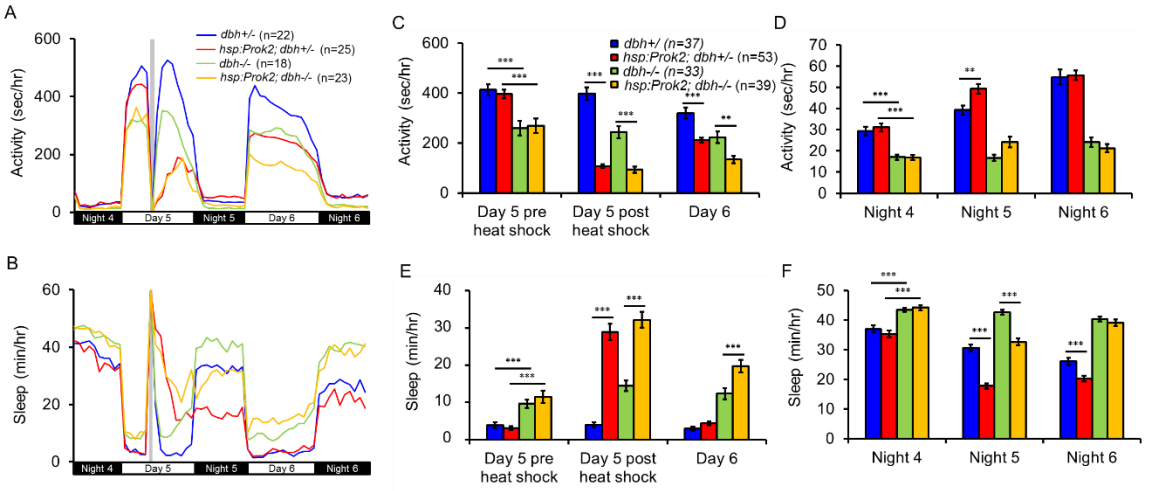

Figure S2.6. Prok2 overexpression phenotype persists in *dbh* mutant larvae. Prok2 was overexpressed in either *dbh*+/- or *dbh* -/- larvae on 5 dpf (grey bar). Larvae in *dbh*-/- background exhibited significantly reduced activity (A, C, D) and increased sleep (B, E, F) than those in *dbh*+/- background both during the day and night as well as before and after heat shock. Prok2 overexpression phenotype persisted in both *dbh*+/- and *dbh*-/- larvae, decreasing activity and increasing sleep during the day, but increasing activity and decreasing sleep at night. Note the lack of difference between *Tg(hsp:Prok2); dbh*+/- and *Tg(hsp:Prok2); dbh*-/- larvae on day 5 post heat shock is likely due to a floor effect. The magnitude of difference between *Tg(hsp:Prok2); dbh*+/- and *Tg(hsp:Prok2); dbh*-/- larvae on day 6 is similar to that between *dbh*+/- and *dbh*-/- larvae. Data from two experiments are shown. Bar graphs show mean ± SEM. n indicates number of larvae analyzed. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA and Tukey's multiple comparison test).

Mutants/Drugs	Affected Pathway
dopamine beta hydroxylase mutant	norepinephrine
histidine decarboxylase mutant	histamine
tryptophan hydroxylase 2 mutant	serotonin
arylalkylamine N-acetyltransferase-2 mutant	melatonin
melatoninm 10 μM	melatonin
dopamine receptor 1 antagonist drug, SCH 23390, 5 μM	dopamine
hypocretin receptor mutant	hypocretin
glucocorticoid receptor mutant	cortisol and glucocorticoids

Table S2.1. List of mutants and small molecules tested for effects on Prok2 overexpression phenotypes.

CHAPTER 3

NEURONAL ACTIVATION AND ABLATION IN FREELY BEHAVING ZEBRAFISH USING TRP CHANNELS

3.1 Abstract

The zebrafish has recently emerged as a useful model system to study neural circuits and behavior, but tools to modulate the activity of genetically specified neurons in freely behaving animals are limited. As a poikilotherm that lives in water, zebrafish are particularly amenable to thermal and pharmacological perturbations. Here we exploit these properties by using transient receptor potential (TRP) channels to inducibly activate and ablate specific neuronal populations in zebrafish larvae using the appropriate chemical and thermal agonists for heterologously expressed TRPV1, TRPM8, and TRPA1. The small size of zebrafish larvae, and the ability to modulate TRP channel function by changing the water temperature or adding the appropriate small molecule to the water, allows for high-throughput behavioral experiments in 96-well plates. We found that expression of TRP channels in Rohon-Beard sensory neurons enables agonist-dependent neuronal activation and startle-like behavioral responses. TRPV1-expressing neurons can be ablated with high concentration of its agonist. Our results show that TRP channels complement existing reagents and expand the scope of behavioral experiments that can be performed using zebrafish.

3.2 Introduction

The complexity of mammalian neural circuits and the difficulty of manipulating them present major challenges in deciphering neural mechanisms that regulate behavior. To overcome these challenges, the zebrafish has recently been developed as a model system to characterize neuronal mechanisms that regulate behavior due to several advantageous features. First, zebrafish embryos and larvae are transparent, which allows

observation and manipulation of neurons in live animals (Arrenberg and Driever, 2013; Friedrich et al., 2013; Leung et al., 2013). Second, morphological and molecular analyses have shown that zebrafish and mammalian brains are similar (Schweitzer et al., 2012; Chiu and Prober, 2013), so neuronal circuits that regulate zebrafish behaviors are likely to be conserved in mammals. For example, the wake-promoting hypocretin system has been shown to be molecularly, anatomically, and functionally conserved in zebrafish (Kaslin et al., 2004; Faraco et al., 2006; Prober et al., 2006; Yokogawa et al., 2007; Elbaz et al., 2012). Third, by the fifth day of development, zebrafish larvae are free-living and exhibit complex behaviors, including hunting and escape responses, allowing detailed behavioral analyses (Orger et al., 2008; Bianco et al., 2011; Patterson et al., 2013; Trivedi and Bollmann, 2013). Fourth, the ability to rapidly raise thousands of zebrafish allows for large-scale screens for genes and neurons that affect behavior (Muto et al., 2005; Wyart et al., 2009). Fifth, zebrafish behaviors can be studied using small molecules that are taken up through the gills or absorbed through the skin (Kokel et al., 2010; Rihel et al., 2010a; Wolman et al., 2011), and zebrafish larvae lack a mature blood brain barrier (Fleming et al., 2013), thus allowing small molecule access to the brain. The zebrafish therefore provides a useful system to characterize neuronal mechanisms that regulate behavior.

A limitation to using zebrafish for these studies is a paucity of tools to manipulate neuronal activity. Several transgenic tools have been developed that allow genetically specified neurons to be stimulated or inhibited. For example, optogenetic tools stimulate or inhibit neurons when illuminated by specific wavelengths of light (Mattis et al., 2011). In principle, the transparency of zebrafish larvae makes them well suited for this

technology. Indeed, optogenetic tools have been used to characterize the roles of specific neurons in sensory and motor control in restrained zebrafish larvae (Douglass et al., 2008; Arrenberg et al., 2009; Wyart et al., 2009). However, the light stimulus elicits a strong behavioral response in unrestrained larvae (Zhu et al., 2009), which is problematic for many behavioral assays, including those used to study sleep (Prober et al., 2006). The confounding effects of light are avoided by tools that modulate neuronal activity in the presence of specific small molecules (Szobota et al., 2007; Arenkiel et al., 2008; Alexander et al., 2009; Magnus et al., 2011) or at specific temperatures (Hamada et al., 2008), but these technologies have not been tested in zebrafish. Here we describe the use of heterologous transient receptor potential (TRP) channels that allow activation or ablation of genetically specified neurons in freely behaving zebrafish larvae.

In this study, we set out to develop tools that could be used to activate genetically-specified neurons in freely behaving zebrafish larvae. We chose to test three transmembrane channels from the TRP cation channel family that are activated by specific small molecules or at specific temperatures in some species, and have been used to stimulate neural activity in mammals (Arenkiel et al., 2008), *Drosophila* (Marella et al., 2006; Hamada et al., 2008) and *C. elegans* (Tobin et al., 2002). We tested the rat TRPV1 channel, which is activated by the small molecule capsaicin (Csn) (Caterina et al., 1997), the rat TRPM8 receptor, which is activated by the small molecule menthol, and the *Crotalus atrox* (rattlesnake) TRPA1 receptor, which is activated at temperatures above 28^{o}C (Gracheva et al., 2010). Importantly, the zebrafish TRPV1 ortholog contains a mutation that renders it insensitive to Csn (Jordt and Julius, 2002; Gau et al., 2013), the zebrafish genome lacks a TRPM8 ortholog, and the two zebrafish TRPA1 paralogs are

not thermosensitive (Prober et al., 2008). Because wild-type zebrafish in principle should not respond to agonists for these heterologous TRP channels, and bath application of small molecules and changes in water temperature can be applied to high-throughput assays of freely behaving zebrafish larvae, these channels could provide a simple yet powerful approach to activate genetically specified neurons without the confounding effects of light on behavior.

3.3 Expression of heterologous TRP channels in zebrafish sensory neurons induces agonist-dependent behavioral phenotypes

To test whether TRP channels can be used to activate genetically specified zebrafish neurons, we used the *islet-1* sensory neuron enhancer (Higashijima et al., 2000) to express rat TRPV1, rattlesnake TRPA1, and rat TRPM8 and in zebrafish trigeminal and Rohon-Beard sensory neurons (Figures 3.1A-3.1C, 3.2A-2C, 3.3A-3.3C), and assayed for behavioral responses to the appropriate channel agonist.

As has previously been shown (Gau et al., 2013), we found that wild-type zebrafish embryos did not exhibit a behavioral response to Csn (Figure 3.1G, S3.1). Similarly, 24 hpf transgenic zebrafish embryos that expressed TRPV1 in sensory neurons exhibited little locomotor activity when treated with DMSO vehicle control or 0.1 μM Csn (Figures 3.1D-3.1I). However, exposure to 0.3 μM Csn or higher concentrations induced robust locomotor activity in transgenic embryos (Figures 3.1D-3.1I), consistent with activation of sensory neurons and similar to the phenotype observed upon activation of sensory neurons using channelrhodopsin-2 (ChR2) (Douglass et al., 2008). At 1 μM, Csn induced a behavioral response in 100% of embryos (Figure 3.1D), with an average 3

second response latency (Figure 3.1F), consisting of very intense locomotor activity lasting for 45 seconds (Figure 3.1E) and less intense activity lasting for up to 2 hours (Figures 3.1G-3.1I). These results suggest that Csn can activate TRPV1-expressing neurons, and thus affect behavior, over long periods of time. Following a 3 or 5 minute washout period in E3 medium, reapplication of Csn elicited a similar behavioral response in 60% and 95% of embryos, respectively (data not shown).

To test the ability of rattlesnake TRPA1 to activate zebrafish neurons, we raised embryos expressing the channel in sensory neurons at temperatures below 28^{o}C, because the channel is activated at and above 28^{o}C (Gracheva et al., 2010). We found that slowly raising the water temperature from 22.5^{o}C to 28.5^{o}C failed to produce a robust behavioral response (data not shown). In contrast, abruptly raising the temperature from 22.5^{o}C to 25.5^{o}C induced a behavioral response in 100% of embryos (Figure 3.2D, S3.2), with an average 1.5 second response latency (Figure 3.2F), consisting of intense locomotor activity lasting for 14 seconds (Figure 3.2E) and less intense activity lasting for up to 2 hours (Figures 3.2G-3.2I). Increasing the temperature from 25^{o}C to 28^{o}C produced similar results (data not shown). In contrast, temperature increases up to 30^{o}C failed to elicit behavioral responses in non-transgenic sibling embryos (Figure S3.3 and data not shown), consistent with previous observations (Prober et al., 2008). The TRPA1-dependent phenotype was remarkably sensitive to temperature, as an increase of as little as 1^{o}C produced a significant behavioral response (Figures 3.2D-3.2F). Similar to TRPV1, the TRPA1-dependent behavioral response could be induced again in 95% of TRPA1-expressing embryos after returning them to 22^{o}C for as little as 2 minutes (data not shown).

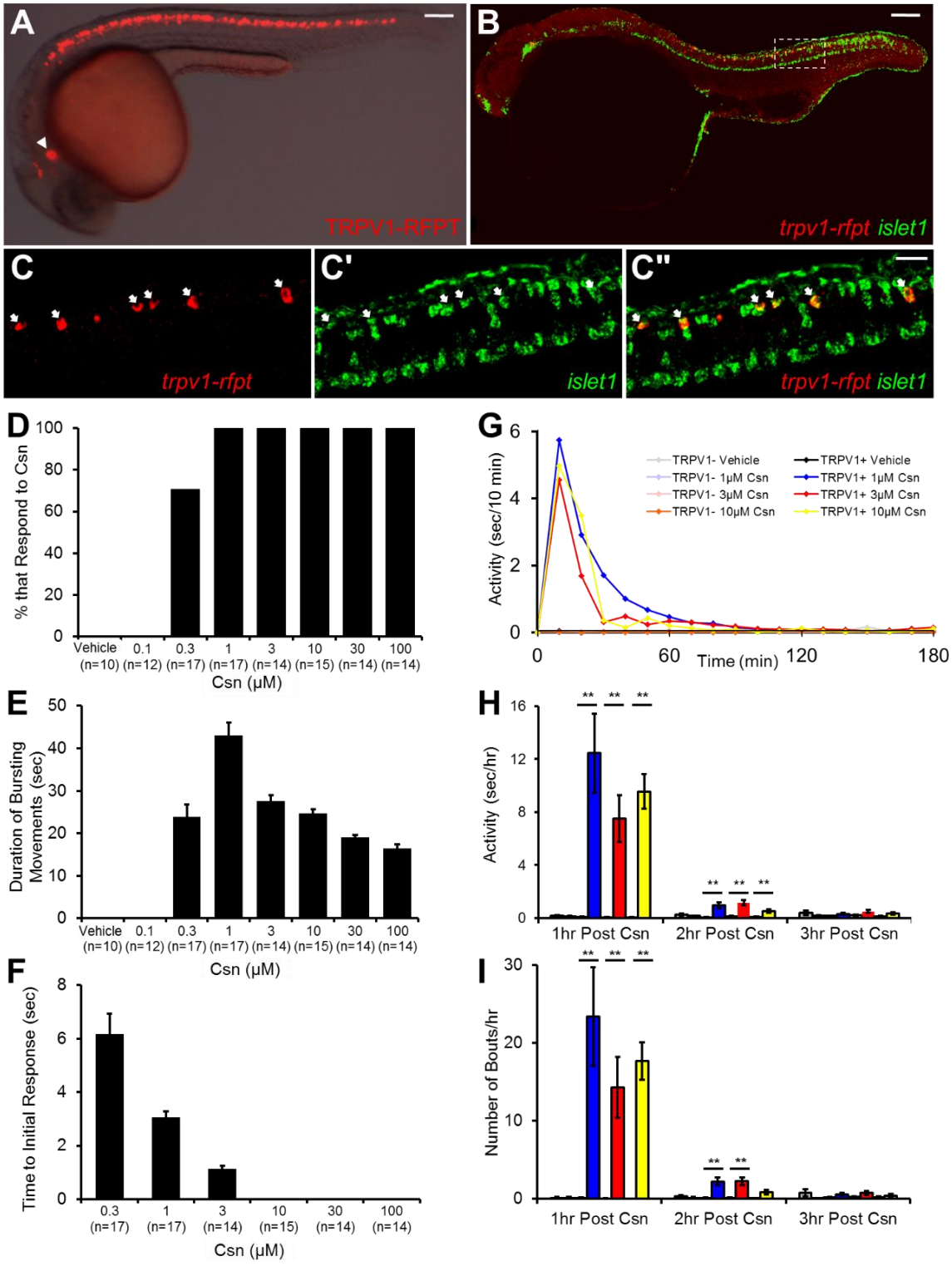
A
TRPV1-RFPT
B
trpv1-rfpt islet1
C
trpv1-rfpt
C'
islet1
C''
trpv1-rfpt islet1
D
% that Respond to Csn
Vehicle (n=10)
0.1 (n=12)
0.3 (n=17)
1 (n=17)
3 (n=14)
10 (n=15)
30 (n=14)
100 (n=14)
Csn (μM)
G
Activity (sec/10 min)
TRPV1- Vehicle
TRPV1- 1μM Csn
TRPV1- 3μM Csn
TRPV1- 10μM Csn
TRPV1+ Vehicle
TRPV1+ 1μM Csn
TRPV1+ 3μM Csn
TRPV1+ 10μM Csn
Time (min)
E
Duration of Bursting Movements (sec)
Csn (μM)
H
Activity (sec/hr)
1hr Post Csn
2hr Post Csn
3hr Post Csn
F
Time to Initial Response (sec)
0.3 (n=17)
1 (n=17)
3 (n=14)
10 (n=15)
30 (n=14)
100 (n=14)
Csn (μM)
I
Number of Bouts/hr
1hr Post Csn
2hr Post Csn
3hr Post Csn

Figure 3.1. Csn induces locomotor activity in embryos expressing TRPV1 in sensory neurons. A 24 hpf *Tg(islet1:GAL4VP16, 4xUAS-TRPV1-RFPT)* embryo exhibits RFPT fluorescence in trigeminal (arrowhead) and Rohon-Beard sensory neurons (A), as confirmed by *trpv1-rfpt* (red) and *islet1* (green) double fluorescent ISH (B). The boxed region in (B) is shown at higher magnification in (C). Arrows indicate Rohon-Beard neurons that express both *trpv1-rfpt* and *islet1*. Scale bars indicate 100 μm (A, B) and 25 μm (C). (D-F) At 24 hpf, transgenic embryos exhibited a dose-dependent behavioral response to Csn, responding to as little as 0.3 μM Csn (D), with a phase of robust bursting movements lasting up to 45 seconds (E) and with a short response latency (F). n indicates number of embryos tested. Non-transgenic siblings did not respond to Csn at any of the concentrations tested (see Figure S3.1 and panel G). (G-I) At 2 dpf, transgenic embryos (bold colors), but not their non-transgenic siblings (faint colors), exhibited a significant increase in locomotor activity (G, H) and in the number of movement bouts (I) in response to 1-10 μM Csn that lasted for up to 2 hours. Mean (G) and mean +/- S.E.M. (E, F, H, I) are shown. ** indicates $p<0.01$ for transgenic embryos compared to their identically treated non-transgenic siblings using the Kruskal-Wallis test and the Steel-Dwass test to correct for multiple comparisons. 20 embryos were tested for each condition (G-I).

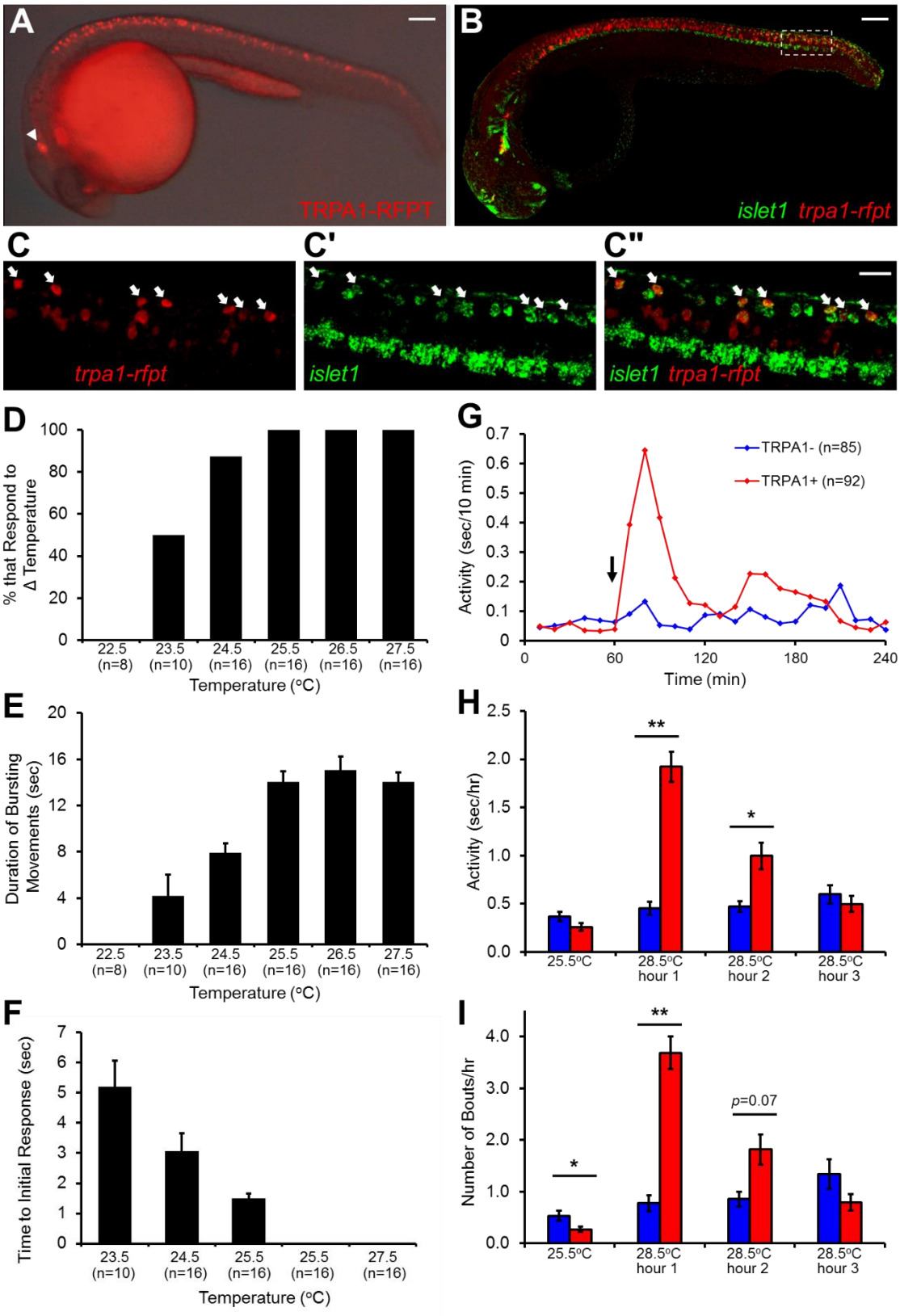
A
TRPA1-RFPT
B
islet1 trpa1-rfpt
C
trpa1-rfpt
C'
islet1
C"
islet1 trpa1-rfpt
D
% that Respond to Δ Temperature
100
80
60
40
20
0
22.5 (n=8)
23.5 (n=10)
24.5 (n=16)
25.5 (n=16)
26.5 (n=16)
27.5 (n=16)
Temperature (°C)
G
Activity (sec/10 min)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TRPA1- (n=85)
TRPA1+ (n=92)
0
60
120
180
240
Time (min)
E
Duration of Bursting Movements (sec)
20
16
12
8
4
0
22.5 (n=8)
23.5 (n=10)
24.5 (n=16)
25.5 (n=16)
26.5 (n=16)
27.5 (n=16)
Temperature (°C)
H
Activity (sec/hr)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
25.5°C
28.5°C hour 1
28.5°C hour 2
28.5°C hour 3
F
Time to Initial Response (sec)
7
6
5
4
3
2
1
0
23.5 (n=10)
24.5 (n=16)
25.5 (n=16)
25.5 (n=16)
27.5 (n=16)
Temperature (°C)
I
Number of Bouts/hr
4.0
3.0
2.0
1.0
0.0
**
*
p=0.07
25.5°C
28.5°C hour 1
28.5°C hour 2
28.5°C hour 3

Figure 3.2. Increased temperatures induce locomotor activity in embryos expressing TRPA1 in sensory neurons. A 24 hpf *Tg(islet1:GAL4VP16, 4xUAS-TRPA1-RFPT)* embryo exhibits RFPT fluorescence in trigeminal (arrowhead) and Rohon-Beard sensory neurons (A), as confirmed by *trpa1-rfpt* (red) and *islet1* (green) double fluorescent ISH (B). The boxed region in (B) is shown at higher magnification in (C). Arrows indicate Rohon-Beard neurons that express both *trpa1-rfpt* and *islet1*. This transgenic line also expresses *trpa1-rfpt* in some spinal cord interneurons. Scale bars indicate 100 μm (A, B) and 25 μm (C). (D-F) Embryos were raised at 26.5ºC and tested at 30 hpf, at which time they were at a similar developmental stage as 24 hpf embryos raised at 28.5ºC. Locomotor activity was induced when the temperature was rapidly increased by 1ºC to 5ºC (D), with a phase of robust bursting movements lasting up to 15 seconds (E) and with a short response latency (F). n indicates number of embryos tested. Wild-type embryos did not exhibit behavioral responses to any of these temperature changes (Figure S3.2 and data not shown). (G-I) At 2 dpf, transgenic embryos (red), but not their non-transgenic siblings (blue), exhibited significant increases in locomotor activity (G, H) and in the number of movement bouts (I) following a change in the water temperature from 25.5ºC to 28.5ºC that lasted for up to 2 hours. The change in water temperature is indicated by an arrow in (G). Mean (G) and mean +/- S.E.M. (E, F, H, I) are shown. * indicates $p<0.05$ and ** indicates $p<0.01$ for transgenic embryos compared to their identically treated non-transgenic siblings using the Wilcoxon rank-sum test. n indicates the number of embryos analyzed.

We also observed robust behavioral responses following application of menthol to embryos expressing rat TRPM8 in sensory neurons (Figures 3.3D-3.3F, S3.3), although higher agonist levels were required compared to TRPV1. For example, application of 100 μM menthol induced a behavioral response in 90% of embryos (Figure 3.3D), with an average 5 second response latency (Figure 3.3F), consisting of intense locomotor activity that lasted for 6 seconds (Figure 3.3E). In contrast, non-transgenic sibling embryos failed to respond to menthol at concentrations up to 1 mM (Figure S3.3 and data not shown). Similar to TRPV1 and TRPA1, the TRPM8-dependent behavioral response to menthol could be repeatedly induced in 90% of embryos following a 2 minute drug washout phase (data not shown). Taken together, our results suggest that TRPV1, TRPA1, and TRPM8 can each be used to activate genetically specified neurons, and thus affect behavior, when stimulated by its specific agonist.

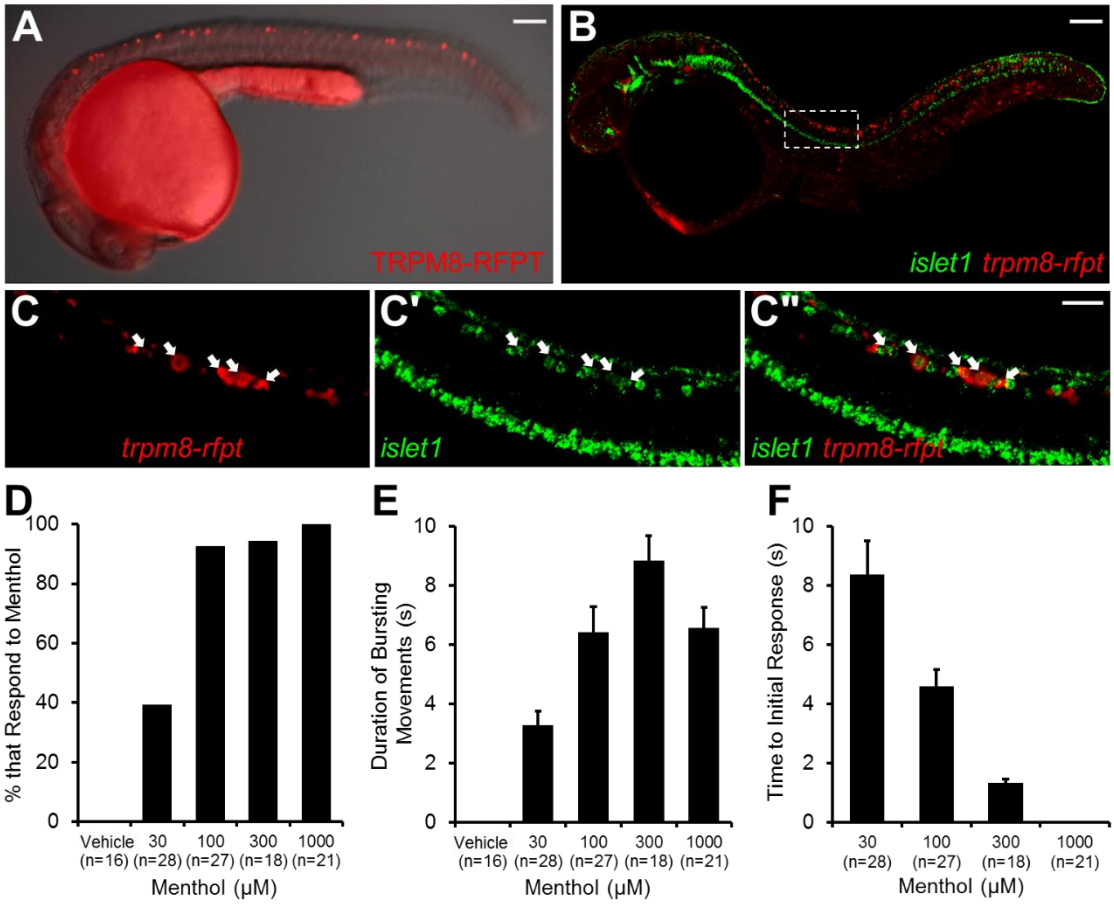

Figure 3.3. Menthol induces locomotor activity in embryos expressing TRPM8 in sensory neurons. A representative 24 hpf wild-type embryo injected with the *Tg(islet1:GAL4VP16, 4xUAS-TRPM8-RFPT)* transgene exhibits TRPM8-RFPT fluorescence in a subset of sensory neurons (A), as confirmed by *trpm8-rfpt* (red) and *islet1* (green) double fluorescent ISH (B). The boxed region in (B) is shown at higher magnification in (C). Arrows indicate Rohon-Beard neurons that express both *trpm8-rfpt* and *islet1*. Scale bars indicate 100 μm (A, B) and 25 μm (C). (D-F) At 24 hpf, transgenic embryos exhibited a dose-dependent behavioral response to menthol, responding to as little as 30 μM menthol (D), with a phase of robust bursting movements lasting up to 9 seconds (E) and with a short response latency (F). Wild-type embryos did not respond to menthol at any of the concentrations tested (Figure S3.3 and data not shown). Mean (D) and mean +/- S.E.M. (E, F) are shown. n indicates number of embryos tested.

3.4 Heterologous TRP channels activate zebrafish sensory neurons in a dose-dependent manner

To more directly test whether TRPV1-expressing sensory neurons are activated by Csn, we assayed neuronal activity in fixed animals using *c-fos in situ* hybridization (ISH) (Baraban et al., 2005) and in real-time using the genetically encoded calcium sensor GCaMP5 (Portugues et al., 2013) following bath application of Csn to intact zebrafish embryos. We observed a dose-dependent increase in the number of *c-fos*-positive TRPV1-expressing sensory neurons, with 90% of these neurons expressing *c-fos* in embryos exposed to 1 μM Csn for 45 minutes (Figures 3.4B, 3.4D). In contrast, we observed no *c-fos* expression in non-transgenic siblings exposed to Csn (Figures 3.4B, 3.4D) or in transgenic embryos exposed to vehicle control (Figures 3.4A, 3.4D). Consistent with these results, Csn induced a dose-dependent increase in GCaMP5 fluorescence in TRPV1-expressing sensory neurons (Figures 3.4E-3.4J). For example, during 440 seconds of exposure to 1 μM Csn, 24% of TRPV1-expressing sensory neurons exhibited transient increases in GCaMP5 fluorescence (Figures 3.4F, 3.4J). At 3 μM Csn, 62% of these neurons responded, with generally larger and longer increases in GCaMP5 fluorescence (Figures 3.4G, 3.4J). At 10 μM Csn, 82% of TRPV1-expressing sensory neurons exhibited very large increases in fluorescence that were sustained until Csn was washed out of the recording chamber (Figures 3.4H, 3.4J). No changes in GCaMP5 fluorescence were observed in transgenic embryos exposed to vehicle control (Figures 3.4E, 3.4J) or in non-transgenic siblings exposed to 10 μM Csn (Figures 3.4I, 3.4J). The average amplitude of calcium transients increased as neurons were exposed to higher Csn concentration (Figures 3.4L). The fractional change in GCaMP response

showed a trend of increase in higher Csn concentration (Figure 3.4M). The average frequency of calcium transients does not show a dose-dependent increase (Figure 3.4K) because 81% of neurons activated by 10 μM Csn showed prolonged calcium transients (Figure 3.4N). These results indicate that TRPV1-expressing sensory neurons, but not wild-type sensory neurons, are activated by Csn in a dose-dependent manner. Our data also suggest that 10 μM Csn causes abnormally high and sustained cytoplasmic calcium levels in TRPV1-expressing neurons that may have deleterious effects. In addition, we observed that 1 μM Csn can repeatedly activate same neurons (Figures 3.5A, 3.5B). 77% of neurons responded to the first Csn application also responded to second Csn application with similar average frequency and amplitude of calcium transients and fractional change in GCaMP response (Figures 3.5C-3.5E). Note there are sometimes additional neurons respond to second Csn application that did not respond to first Csn application.

We next tested whether Rohon-Beard sensory neurons expressing TRPA1 or TRPM8 can be activated by their respective agonists using *c-fos* ISH. We found that 44% of TRPA1-expressing sensory neurons expressed *c-fos* following a temperature shift from 25^{o}C to 28^{o}C (Figures 3.6B, 3.6D), while no *c-fos* expression was observed in identically treated non-transgenic siblings (Figures 3.6C, 3.6D). We observed *c-fos* expression in only 3% of TRPA1-expressing sensory neurons in embryos maintained at 25^{o}C (Figures 3.6A, 3.6D). Similarly, 65% of TRPM8-expressing sensory neurons expressed *c-fos* following exposure to 100 μM menthol (Figures 3.6F, 3.6H), while no *c-fos* expression was observed in TRPM8-expressing sensory neurons exposed to vehicle control (Figures 3.6E, 3.6H) or in non-TRPM8-expressing sensory neurons exposed to 100 μM menthol

(Figures 3.6G, 3.6H). We conclude that TRPA1- and TRPM8-expressing sensory neurons, but not wild-type sensory neurons, are activated by increased temperature and menthol, respectively.

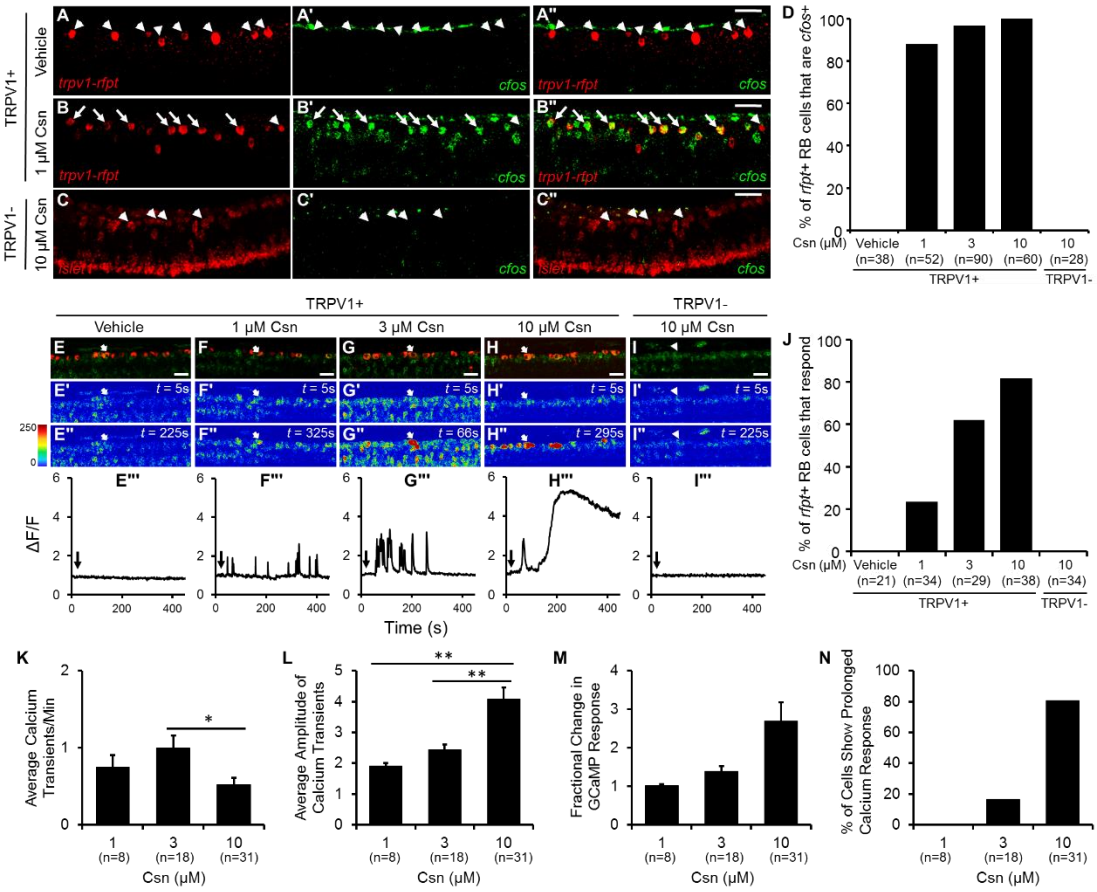

Figure 3.4. Csn activates TRPV1-expressing Rohon-Beard neurons. (A-C) Representative double fluorescent ISH images showing that *c-fos* expression is induced in *trpv1-rfpt*-expressing Rohon-Beard neurons in transgenic embryos exposed to 1 μM Csn for 45 minutes (B), but not in transgenic siblings exposed to the vehicle control (A) or non-transgenic siblings exposed to 10 μM Csn (C). *islet1* expression was used to identify Rohon-Beard neurons in non-transgenic embryos in (C). Arrows and arrowheads indicate Rohon-Beard sensory neurons that do and do not express *c-fos*, respectively. (D) Quantification of the percentage of *trpv1-rfpt*-expressing Rohon-Beard neurons that express *c-fos* following the indicated treatments. (E-I) Representative images showing TRPV1-RFPT expressing neurons (E-H) and GCaMP5 fluorescence before (E'-I') and after (E"-I") addition of Csn at the indicated concentrations. Rohon-Beard neurons were identified in non-transgenic controls based on their morphology and location using basal

GCaMP5 fluorescence (I). White arrows indicate Rohon-Beard neurons whose fluorescence intensities are quantified in panels (E”’-I”’). Time of Csn addition is indicated by black arrows (E”’-I”’). (J) Quantification of the percentage of *trpv1-rfpt*-expressing Rohon-Beard neurons that show increased GCaMP5 fluorescence following exposure to Csn at the indicated concentrations. n indicates number of neurons analyzed. (K-N) Quantification of average calcium transients/min, average amplitude of calcium transients, fractional change in GCaMP response, and % of cells show prolonged calcium response of the cells that showed at least one calcium transient (defined as at least 50% increase in $\Delta F/F_0$). Note that average calcium transients for 10 μM are lower because most neurons show prolonged calcium response. Scale bars=25 μm. 3 animals were used were used for each condition in (D) and (J). Animals used: (D) n=4 for vehicle, n=6 for TRPV1+ in 1 μM Csn, n=7 for TRPV1+ in 3 μM Csn, n=7 for TRPV1+ in 10 μM Csn; (J) n=3 for each condition.

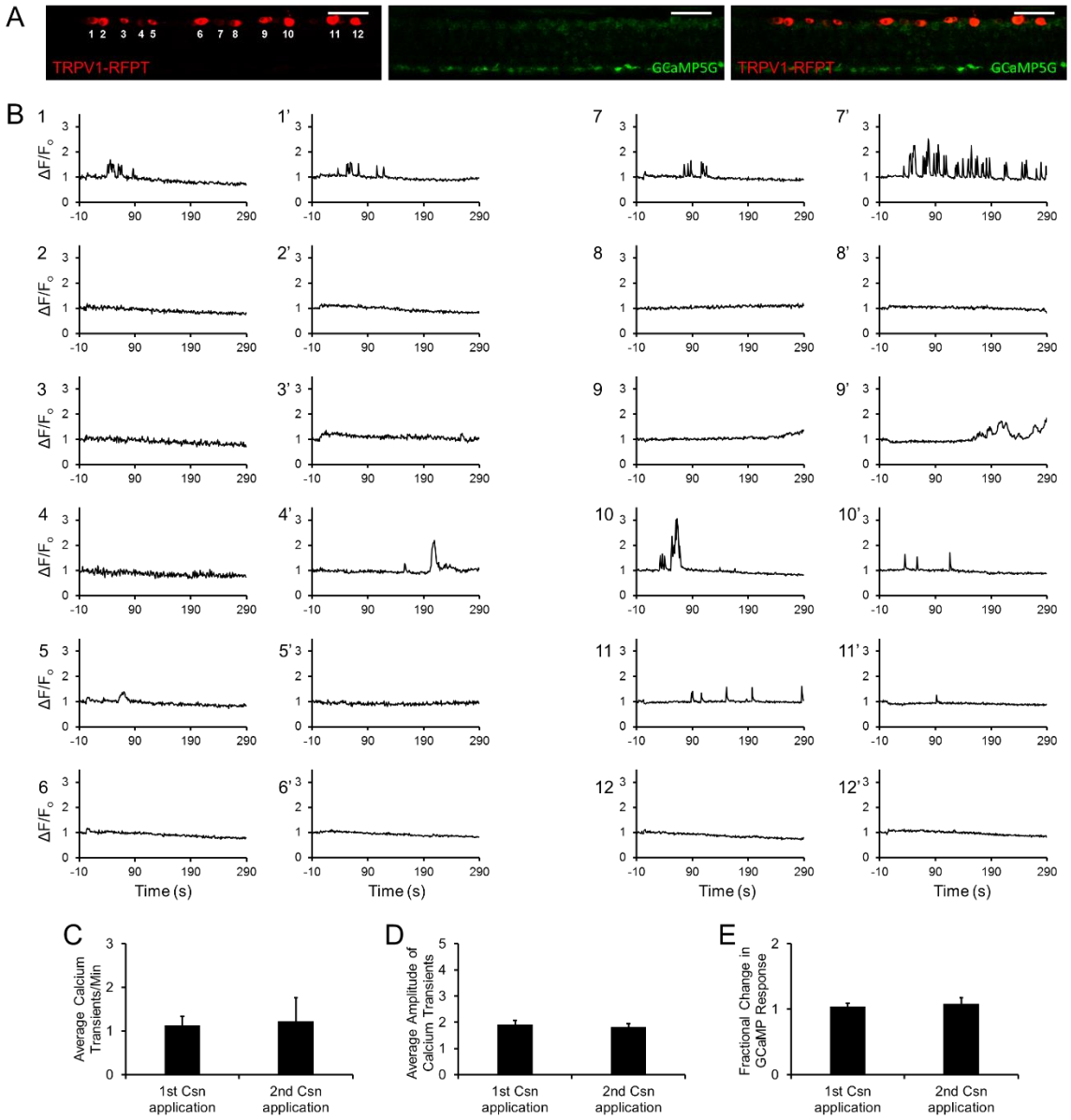

Figure 3.5. Csn can repeatedly induce GCaMP response in TRPV1+ neurons. (A) Representative images showing TRPV1-RFPT expressing neurons and GCaMP5 fluorescence before addition of Csn. (B) Change in fluorescence ($\Delta F/F_0$) was plotted for every cells in the field of view for both first (1-12) and second round (1‘-12’) of Csn application in (A). (C-E) Quantification of average calcium transients/min, average amplitude of calcium transients, and fractional change in GCaMP response of 10 neurons that responded to both application of Csn. 3 animals were used for (C)-(E). Total number of cells that responded both times = 10. Scale bar = 50 μm.

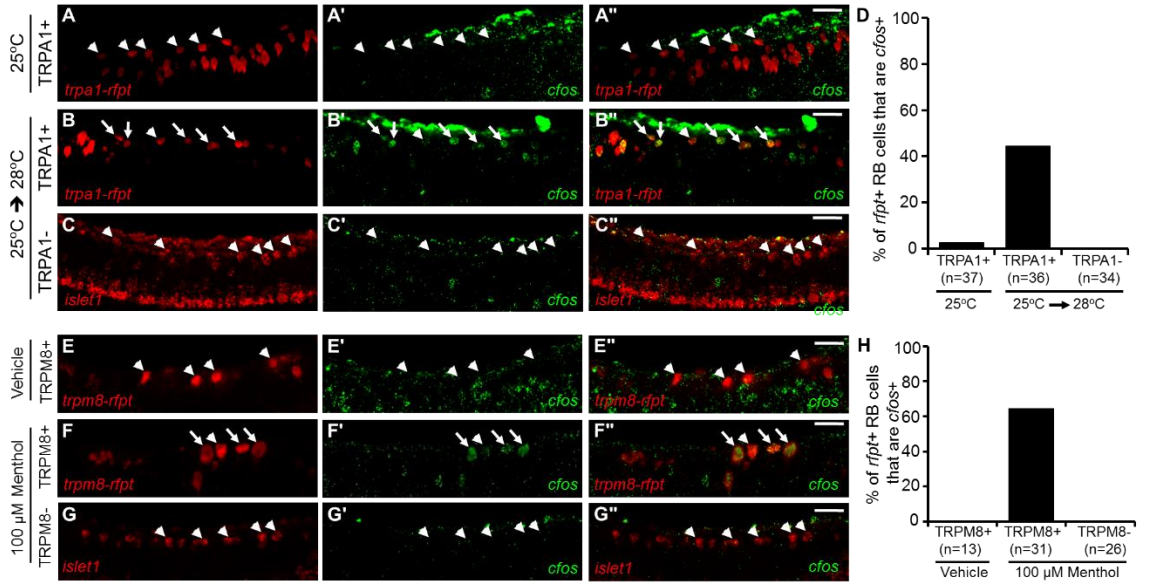

Figure 3.6. Increased temperature activates TRPA1-expressing Rohon-Beard sensory neurons and menthol activates TRPM8-expressing Rohon-Beard neurons. (A-C) Representative images showing that *c-fos* expression is induced in *trpa1-rfpt*-expressing Rohon-Beard neurons in transgenic embryos by 45 minutes after a change in water temperature from 25ºC to 28ºC (B), but not in non-transgenic siblings subjected to the temperature change (C) or in transgenic siblings maintained at 25ºC (A). (D) Quantification of the percentage of *trpa1-rfpt*-expressing Rohon-Beard neurons that express *c-fos* following a 25ºC to 28ºC temperature change. (E-G) Representative images showing that *c-fos* expression is induced in *trpm8-rfpt*-expressing Rohon-Beard neurons in transgenic embryos exposed to 100 μM menthol for 45 minutes (F), but not in transgenic siblings exposed to the vehicle control (E) or non-transgenic siblings exposed to 100 μM menthol (G). (H) Quantification of the percentage of *trpm8-rfpt*-expressing Rohon-Beard neurons that express *c-fos* following exposure to 100 μM menthol. *islet1* expression was used to identify Rohon-Beard neurons in non-transgenic embryos (C, G). Arrows and arrowheads indicate Rohon-Beard sensory neurons that do and do not express *c-fos*, respectively. n indicates number of neurons analyzed. Scale bars=25 μm. Animals used: (D) n=3 for each condition; (H) n=4 for vehicle, n=8 for TRPM8+ in 100 μM menthol, n=3 for TRPM8- in 100 μM menthol.

3.5 Prolonged exposure to high Csn levels causes TRPV1-dependent cell death

In addition to short-term behavioral experiments, we also tested the effect of activating each TRP channel over prolonged periods of time. By measuring TRPV1-RFPT fluorescence as a proxy for cell number, we found that treating embryos containing

TRPV1-expressing sensory neurons with 1 μM Csn for up to 24 hours had no effect on the number of TRPV1-expressing neurons compared to controls (Figures 3.7A, 3.7B, 3.7D). In contrast, the number of TRPV1-expressing neurons was dramatically reduced by treatment with 10 μM Csn for 10 hours, and virtually all TRPV1-expressing neurons were absent after 24 hours of exposure (Figures 3.7C, 3.7D). As a negative control, we used Et(*e1b:GAL4VP16*)s1102t; Tg(14x*UAS-EGFP-Aequorin*) double transgenic embryos to express EGFP, but not TRPV1, in Rohon-Beard sensory neurons. The number of these neurons was unaffected by exposure to 10 μM Csn for 24 hours (Figures 3.7E-3.7G), indicating that Csn-mediated cell ablation requires TRPV1-expression. Importantly, embryos exposed to 10 μM Csn for up to 48 hours developed normally, appeared healthy and exhibited normal patterns and levels of locomotor activity. To confirm that the disappearance of RFPT fluorescence is a result of cell death and not suppression in transgene expression, we performed acridine orange staining, a vital dye that is often used as a marker for apoptosis in larval zebrafish (Tucker and Lardelli, 2007; Eimon, 2014). We observed acridine orange staining only in TRPV1+ neurons treated with 10 μM Csn (Figure 3.8). In contrast to TRPV1, the number of TRPA1-expressing sensory neurons was not reduced in embryos incubated for 24 hours at 28^{o}C compared to embryos maintained at 25^{o}C (data not shown). Similarly, exposure to 100 μM menthol for up to 24 hours had no effect on the survival of TRPM8-expressing sensory neurons (data not shown). Based on these observations, we conclude that strong and prolonged activation of TRPV1 results in cell death, thus providing a novel and robust method for genetically targeted and inducible neuronal ablation in zebrafish.

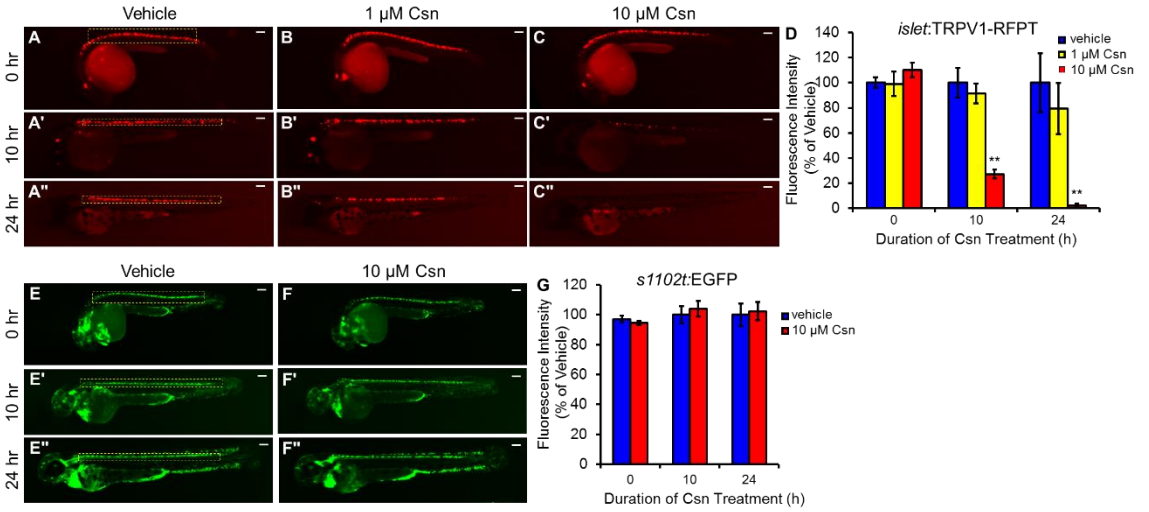

Figure 3.7. High Csn levels ablate TRPV1-expressing Rohon-Beard neurons. (A-C) Representative images of *Tg(islet1:GAL4VP16, 4xUAS-TRPV1-RFPT)* transgenic embryos incubated in vehicle control, 1 μM Csn or 10 μM Csn for the indicated time periods. RFPT fluorescence is dramatically reduced and essentially absent after 10 hr and 24 hr, respectively, in 10 μM Csn, (C). Incubation in 1 μM Csn has no significant effect on RFPT fluorescence (B) compared to embryos treated with the vehicle control (A). (D) Quantification of RFPT fluorescence intensity for the conditions shown in (A-C). (E-F) Representative images of Et(*e1b:GAL4VP16*)s1102t; Tg(*UAS-EGFP-Aequorin*) double transgenic embryos incubated in vehicle control or 10 μM Csn for the indicated time periods. EGFP fluorescence is unaffected by Csn treatment. (G) Quantification of EGFP fluorescence intensity for the conditions shown in (E-F). The region of interest used to quantify fluorescence at each time point is indicated by the yellow boxes in (A) and (E). Mean +/- S.E.M. is shown in (D) and (G). ** indicates $p<0.01$ by Tukey's HSD test. Six embryos were analyzed for each condition. Scale bars=100 μm.

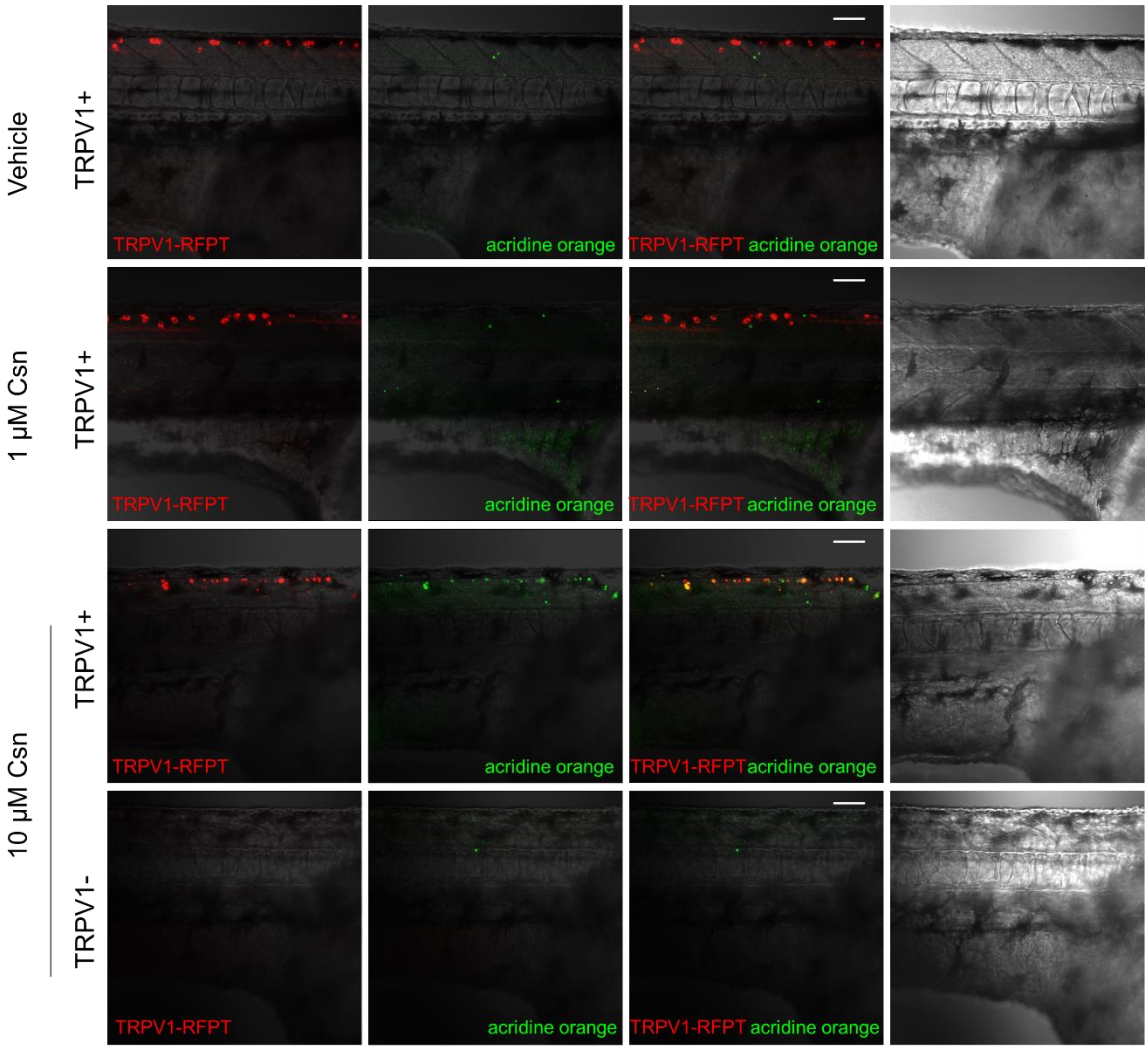

Figure 3.8. High Csn concentration induces cell death. Representative images showing acridine orange is observed only in TRPV1+ neurons treated with 10 µM Csn. No acridine orange signal is observed along the spinal cord in TRPV1- larvae. Scale bar = 50 µm.

3.6 Discussion

In this study, we validate the use of TRP channels for specific activation and ablation of neurons in freely behaving zebrafish. This technology offers several advantages over currently available technologies. First, these pharmaco- and thermo-genetic tools can be used in freely behaving zebrafish larvae without disruption of light-

dependent behaviors such as sleep. While using small molecules and temperature change to activate TRP channels lacks the millisecond temporal resolution of optogenetic tools, fine scale neuronal activation may not be necessary for behaviors that are regulated over relatively long time scales, such as sleep/wake behaviors. Indeed, TRPA1 has become the method of choice for long-term activation of genetically specified neurons in *Drosophila* (Bernstein et al., 2012). While long-term activation of TRP channels may lead to desensitization and a cessation of neuronal activity, our experiments suggest that this can be rapidly reversed by washing out the small molecule or lowering the temperature, which is easy to perform in high-throughput behavior assays.

Second, TRP channels can play a complementary role with existing genetic methods that report neuronal activity by release of light. For example, aequorin, a luminescent Ca^{2+} indicator, has recently been shown to report the activity of neurons in freely behaving zebrafish (Naumann et al., 2010). Additionally, TRP channels can be used in combination with fluorescent Ca^{2+} indicators such as GCaMP, whose use in combination with optogenetic tools such as ChR2 is complicated by the requirement of blue light to both activate ChR2 and excite GCaMP fluorescence.

Third, the single channel conductance of TRP channels is 1000-fold larger than for ChR2 (Bernstein et al., 2012), so TRP channels can drive neuronal activity at lower expression levels, and weak promoters that do not achieve sufficiently high levels of ChR2 expression to stimulate neuronal activity may be sufficient for TRP channels. Lower expression also minimizes potential toxicity resulting from expression of a heterologous protein.

Fourth, because the three TRP channels that we have described are activated by

distinct chemical or thermal agonists, they can be used to independently stimulate and/or ablate three distinct neuronal populations simultaneously. This feature will facilitate studies of how different neuronal populations interact to regulate behavior.

Fifth, TRPV1-mediated cell ablation is faster, more robust and lacks the non-specific toxicity associated with the standard approach used to ablate cells in zebrafish, in which a nitroreductase transgene converts the inert prodrug metronidazole into a cell-autonomous toxin (Curado et al., 2007; Pisharath et al., 2007), although enhanced versions of nitroreductase have recently been described (Mathias et al., 2014; Tabor et al., 2014). As a result, TRPV1 will be particularly useful for neuronal populations that are not ablated by nitroreductase due to insufficiently strong transgene expression.

Sixth, the ability of the TRPV1 to activate or ablate neurons depending on agonist concentration will allow for efficient screens for neurons that are necessary and/or sufficient for various behavioral phenotypes. More generally, it will be interesting to determine whether TRP channels can also be used to ablate and/or modulate calcium levels in non-neuronal cell types.

Finally, we note that zebrafish are uniquely well-suited among standard vertebrate model organisms for using TRP channels to stimulate neuronal activity. Small molecules can be administered to zebrafish larvae by simply adding them to the water, where the compounds are rapidly taken up and can access neurons deep within the brain. Furthermore, the 28^{o}C activation temperature of rattlesnake TRPA1 is within the normal temperature range of zebrafish larva, yet is high enough that zebrafish larvae can be raised and subjected to behavioral assays at slightly lower temperatures that do not activate the channel. In contrast, administering small molecules or heating neurons in the

mammalian brain is challenging, and all known TRP channels are either activated at temperatures below the 37^{o}C body temperature of mammals or at higher temperatures that cause tissue damage (Bernstein et al., 2012). We therefore expect that TRP channels will be useful tools for studying zebrafish neuronal circuits and behavior.

3.7 Materials and Methods

Transgenic zebrafish. The rat TRPV1 channel (NM_031982) containing the E600K mutation, which increases sensitivity to capsaicin (Csn) by over 10-fold (Jordt et al., 2000), the rat TRPM8 (NM_134371) channel, and the Crotalus atrox (rattlesnake) TRPA1 (GU562967) channel were each fused to TagRFPT (Shaner et al., 2008) at their C-termini. For expression in trigeminal and Rohon-Beard sensory neurons, the 4 kb *islet1* sensory neuron specific enhancer (Higashijima et al., 2000) was cloned upstream of the GAL4VP16 transcriptional activator (Koster and E, 2001), followed by 4xUAS:E1b minimal promoter-TRP channel-RFPT. The open reading frame was followed by an SV40 polyA sequence and cassette was flanked by ISce1 meganuclease sites and Tol2 transposase arms. The *Tg(islet1:GAL4VP16, 4xUAS:TRPV1-RFPT)* and *Tg(islet1:GAL4VP16, 4xUAS:TRPA1-RFPT)* transgenic lines were generated using the ISce1 approach (Thermes et al., 2002). TRPM8 experiments used transient injection of an *islet1:GAL4VP16, 4xUAS:TRPM8-RFPT* transgene because we did not generate a stable transgenic line. The *Tg(elavl3:GCaMP5G)* (Portugues et al., 2013), Et(*e1b:GAL4VP16*)s1102t (Scott and Baier, 2009) and *Tg(14xUAS:EGFP-Aequorin*) (Naumann et al., 2010) transgenic lines have previously been described.

***in situ* hybridization and immunohistochemistry.** Zebrafish samples were fixed in 4% paraformaldehyde/PBS for 12-16 hours at room temperature. Double fluorescent *in situ* hybridizations (ISH) were performed using digoxigenin (DIG) and 2,4-dinitrophenol (DNP)-labeled riboprobes and the TSA Plus DIG and DNP System (PerkinElmer, Wellesley, MA). An 818 bp template for the *c-fos* probe was amplified from a larval zebrafish cDNA library using the primers 5'- CAGCTCCACCACAGTGAAGA-3' and 5'- TGCAAACAATTCGCAAGTTC-3'. A 735 bp template for the *rfpt* probe was amplified from the TRPV1-RFPT plasmid using the primers 5'-ATGGTGTCTAAGGGCGAAGA-3' and 5'-TTACTTGTACAGCTCGTCCATG-3'. The islet-1 probe was generated as described (Higashijima et al., 2000). Samples were mounted in 50% glycerol in PBS, and imaged on a Zeiss LSM 780 laser-scanning confocal microscope with 488 nm and 561nm lasers and 10x, 25x and 40x objectives.

Small molecule treatment. Frozen stock solutions of 100 mM Csn (M2028, Sigma) and 500 mM Menthol (M2772, Sigma) were prepared by dissolving the compounds in dimethyl sulfide (DMSO). Working concentrations were prepared just prior to embryo treatment by diluting the stock solutions into E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.4). All treatment conditions contained a final concentration of 0.2% DMSO.

Temperature-dependent activation of TRPA1. *Tg(islet1*:Gal4VP16, *UAS:*TRPA1-RFPT) transgenic larvae were raised in E3 medium at 22.5^{o}C or 26.5^{o}C to avoid

activating the TRPA1 channel. Embryos were transferred to E3 medium at temperatures up to 28.5^{o}C to activate the channel.

TRPV1-mediated cell ablation. For TRPV1-mediated Rohon-Beard cell ablation, 28 hours post-fertilization (hpf) embryos were dechorionated and treated with 1 μM or 10 μM Csn for 24 hours. Embryos were imaged just before Csn addition, and at 10 and 24 hours after Csn addition. At each time point, each embryo was anesthetized with 0.03% tricaine and oriented on its side. Fluorescent images were acquired using a dsRed filter on a fluorescent stereomicroscope (M250c, Leica Microsystems Inc.) and a color CCD camera (DFC310FX, Leica Microsystems Inc.). Using ImageJ, fluorescence intensity was quantified in a 200 μm x 1.6 mm (28 hpf) or 100 μm x 2 mm (34 hpf and 48 hpf) region of interest that encompassed the dorsal spinal cord and most Rohon-Beard sensory neurons. Fluorescence intensity was calculated by subtracting background fluorescence from the measured fluorescence.

Calcium imaging. Embryos at 2 days post-fertilization (dpf) were paralyzed with 1 mg/ml α-bungarotoxin (2133, Fisher Scientific) dissolved in E3 medium and mounted on their side in 0.8% low melting point agarose in a 35 x 10 mm culture dish, and covered with 3 mL E3 medium. *Tg(elavl3:GCaMP5G)* fluorescence was imaged using a Zeiss 780 confocal microscope with 20x dipping objective. Samples were excited with a 488 nm laser and emitted light was collected through a 493-569 nm filter. For one-time activation by Csn experiments, images were acquired at 1 frame/second for 10 seconds before and 440 seconds after Csn application. Frame acquisition time was approximately

900 milliseconds. One mL of Csn at 4 μM, 12 μM, or 40 μM was added to the side of culture dish for a final concentration of 1 μM, 3 μM, or 10 μM, respectively. For repeated activation experiments, images were acquired at 1 frame/second for 10 seconds before and 290 seconds after Csn application. One mL of Csn at 4 μM was first added to the side of the dish for a final concentration of 1 μM. After imaging, Csn solution in the dish was washed out with E3 3 times for 5 minutes each. Same larva was imaged again using the same setting. One mL of Csn at 4 μM was again added to the side of the dish for a final concentration of 1 μM. GCaMP signal was imaged for 10 seconds before and 290 seconds after Csn application. Fluorescence intensities were measured by drawing regions of interest around Rohon-Beard neurons using ImageJ. Change in fluorescence ($\Delta F/F_0$) was calculated as the fluorescence minus the initial fluorescence (defined as the mean fluorescence for the first 10 images) divided by initial fluorescence. A calcium transient is defined as at least 50% increase in $\Delta F/F_0$ from baseline. Average frequency of calcium transients was calculated by averaging the total number of calcium transients in each cell for each Csn concentration. Average amplitude of calcium transients was calculated by averaging the peak $\Delta F/Fo$ value of all calcium transients in each cell for each Csn concentration. Fractional changes in GCaMP response were calculated by averaging $\Delta F/Fo$ over 290s after Csn application for each cell. Prolonged calcium response is defined as any calcium transients lasting for more than 30s.

Locomotor activity behavioral assay. Larval zebrafish were raised on a 14 hour:10 hour light:dark cycle at 28.5°C (except for TRPA1+ larvae which were raised at 22^{o}C) with lights on at 9 am and off at 11 pm. Individual larvae were placed into each well of a 96-

well plate (7701-1651, Whatman) containing 650 μL E3 embryo medium. 2 dpf larvae were transferred into E3 medium containing Csn or vehicle control immediately before the start of behavioral recording at 12 pm. Locomotor activity was monitored using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch Monochrome camera (Dragonfly 2, Point Grey) fitted with a variable-focus megapixel lens (M5018-MP, Computar) and infrared filter. The movement of each larva was recorded at 15 Hz, with an integration time of 1 second, using the quantization mode. One computer collected data from two cameras simultaneously. 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 (except for TRPA1+ larvae which were maintained at 25°C). The parameters used for detection were: detection threshold, 15; burst, 29; freeze, 3. Data were processed using custom PERL and Matlab (The Mathworks, Inc) scripts. Definitions of sleep/wake parameters were described previously (Prober et al., 2006). Statistical tests were performed using JMP and Matlab.

Acridine orange staining. 24 hpf embryos were incubated in vehicle control, 1 μM or 10 μM Csn for 8 hours. Then the embryos were transferred to 5 μg/mL acridine orange dissolved in E3 for 20 minutes in dark, followed with two 5-minute washes with E3. Images was acquired with a Zeiss 780 confocal microscope with 20x dipping objective using following settings: acridine orange – excited with a 488 nm laser and emitted light was collected through a 493-544 nm filter; TRPV1-RPPT – excited with a 561 nm laser

and emitted light was collected through a 624-659 nm filter. The bright field overlay show the position of the Rohon-Beard neurons along the spinal cord.

Supplemental figures

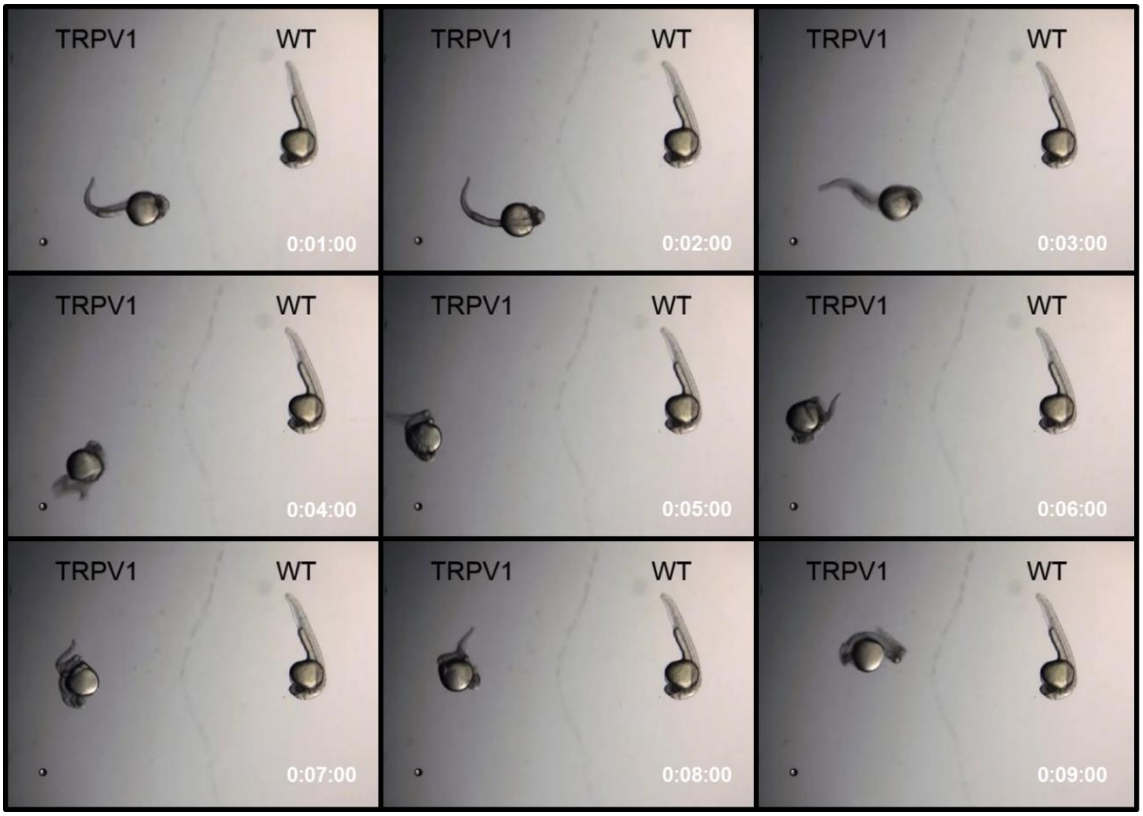

Figure S3.1. Image series showing behavioral response to Csn activation of TRPV1-expressing sensory neurons at 24hpf. *Tg(islet1:GAL4VP16, 4xUAS-TRPV1-RFPT)* embryos, but not their non-transgenic siblings, exhibit a robust behavioral response when immersed in 1 μM Csn. Images are captured at one second intervals after larvae were added to the dish.

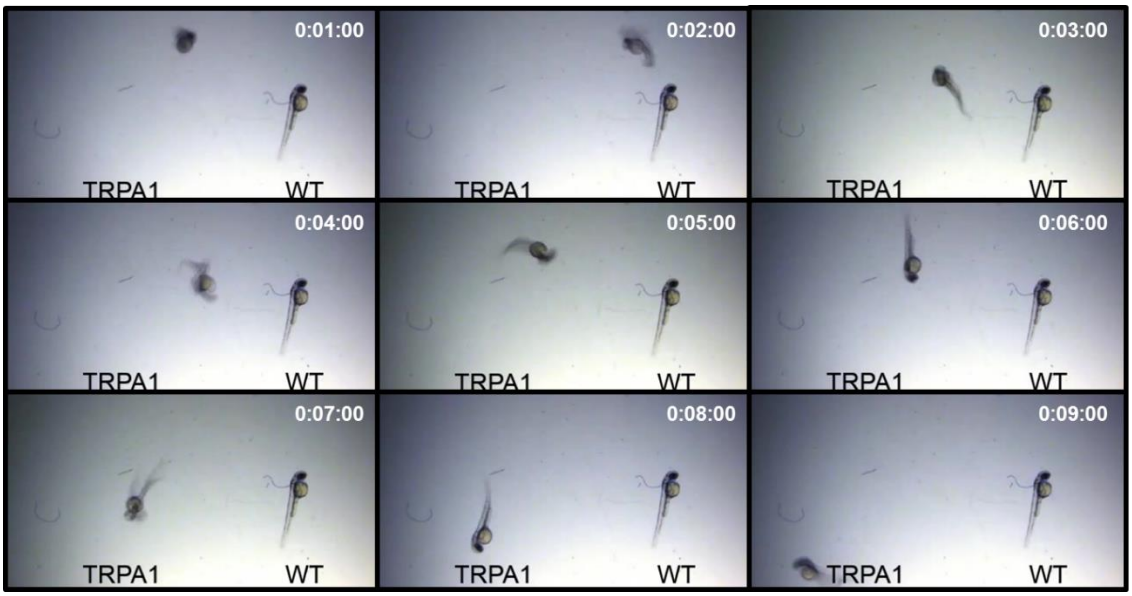

Figure S3.2. Image series showing behavioral response to thermal activation of TRPA1-expressing sensory neurons at 48 hpf raised at 22 ºC. *Tg(islet1:GAL4VP16, 4xUAS-TRPA1-RFPT)* embryos, but not their non-transgenic siblings, exhibit a robust behavioral response when immersed in 27.5ºC water after being maintained at 22.5ºC. Images are captured at one second intervals after larvae were added to the dish.

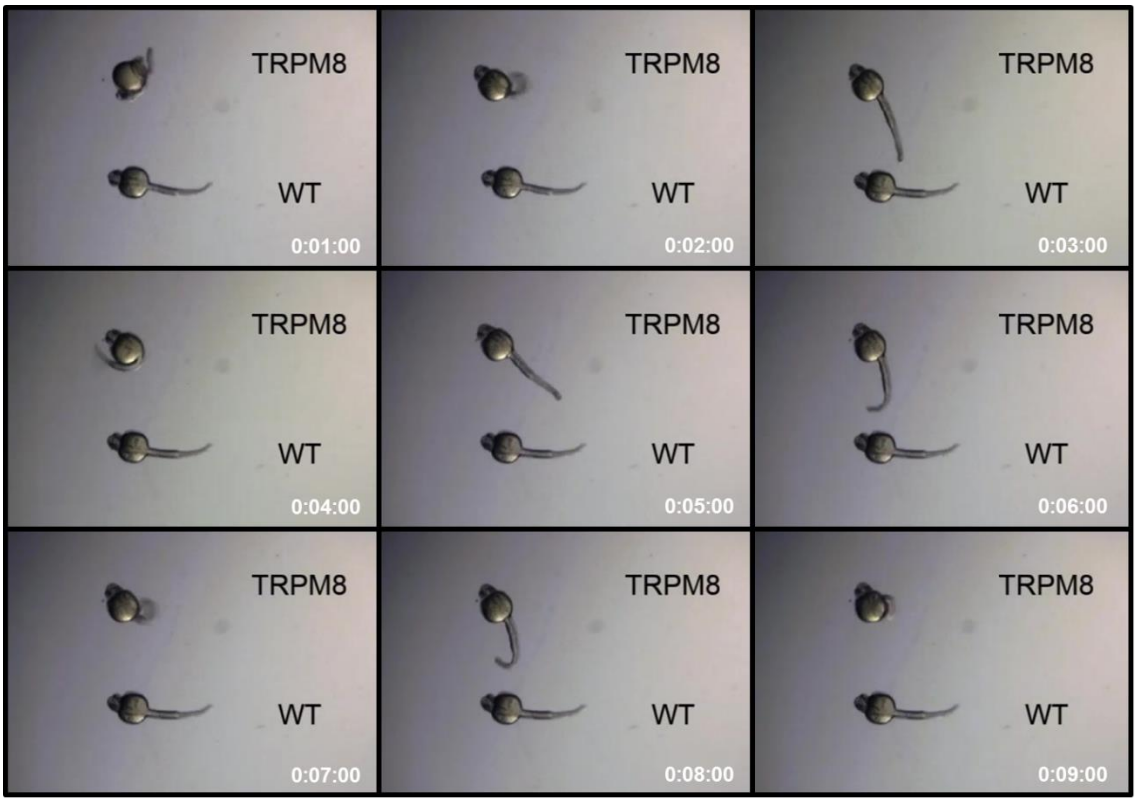

Figure S3.3. Image series showing behavioral response to menthol activation of TRPM8-expressing sensory neurons at 24 hpf. Wild-type embryos injected with an *islet1:*GAL4VP16, *4xUAS*-TRPM8-RFPT transgene, but not their uninjected siblings, exhibit a robust behavioral response when immersed in 300 μM menthol. Images are captured at one second intervals after larvae were added to the dish.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This thesis uses larval zebrafish as a model to study circadian regulation of sleep in diurnal vertebrates. Chapter 2 characterizes the function of Prok2, a proposed circadian output molecule, in larval zebrafish (Cheng et al., 2002; Zhou and Cheng, 2005). The motivation behind the study is to better understand the role of Prok2 in regulating sleep/wake behavior in diurnal vertebrates, and to compare its function to that in nocturnal animals.

I show that in larval zebrafish, Prok2 is expressed exclusively at the ventral hypothalamus, a region homologous to the mammalian SCN. Similar to nocturnal animals (Cheng et al., 2002; Li et al., 2006), Prok2 is required for normal sleep/wake behavior in larval zebrafish, and Prok2 overexpression is sufficient to modulate sleep/wake states. In addition, I discovered that Prok2 overexpression suppresses the predominant behavior induced by the lighting condition, and does not require an internal circadian clock. Furthermore, *prok2-/-* larvae exhibit hyperactivity and reduced sleep during the day. Combined with the genetic gain of function experiment, these results support a novel function of Prok2 acting as a homeostatic regulator to modulate and prevent hyperactivity in light in diurnal vertebrates.

Unlike nocturnal rodents, *prok2-/-* larvae do not show attenuated circadian rhythmicity, as measured by sleep/wake behavior after removing external light cues. This discrepancy suggests that Prok2 may function differently in diurnal vs nocturnal animals. The role of Prok2 in regulation of circadian rhythm and sleep in humans has been reported in three studies. Two earlier studies reported that humans with a mutation in Prok2 experience sleep disorders, but did not specify which kind of sleep disorders (Dodé

et al., 2006; Cole et al., 2008). A recent study by Balasubramanina et al., reported that human patients with a loss of function mutation in Prok2 did not show abnormalities in central circadian rhythmicity, as measured by circulating cortisol and melatonin level, but exhibited impaired performance at the psychomotor vigilance task (Balasubramanian et al., 2014), which is often correlated to sleep deficit (Dinges et al., 1997; Van Dongen and Dinges, 2005). This observation is similar to the phenotype of *prok2-/-* larvae. *prok2-/-* larvae did not show changes in circadian rhythmicity measured by sleep/wake behavior after transitioning from regular light dark cycle into constant dark, but exhibit a change in homeostatic regulation of sleep during the day. One potential caveat to this explanation is that the measurement of central circadian rhythmicity is different in humans and zebrafish larvae. A more detailed characterization of sleep/wake behavior in human patients is needed to answer how sleep/wake behavior is affected. It is possible Prok2 mediates circadian rhythmicity in sleep/wake behavior but not in the release of neuro-endocrine molecules in humans. Nonetheless, the data on human patients so far agrees with the phenotype we observed in *prok2-/-* larvae, suggesting that larval zebrafish, being a diurnal vertebrate, may be a more appropriate model for studying circadian regulation of sleep for humans. In addition, this study also points out the potential difference of the function of circadian regulators between diurnal and nocturnal animals. Given the under-representation of diurnal vertebrates used in sleep research, larval zebrafish may become an attractive model for studying circadian regulation of sleep in humans.

Although Prok2 has been shown to bind to two receptors *in vitro* (Lin et al., 2002), Prok2 overexpression phenotype is specifically mediated by Prokr2 *in vivo*. *prokr2* is expressed in several known sleep/wake centers in larval zebrafish, including the

noradrenergic locus coeruleus and histaminergic tuberomammillary nucleus. However, the Prok2 overexpression phenotype persisted in mutants that lack epinephrine, histamine or several other known sleep regulators (Table S2.1.), suggesting that Prok2 does not affect sleep via these neuromodulators. It remains possible that blocking more than one of these pathways simultaneously could inhibit the effects of Prok2 overexpression on sleep. Alternatively, Prok2 may act via known sleep regulatory pathways that were not tested or via unknown sleep regulators. Further characterization of Prok2 signaling or *prok2*-expressing neurons could answer these questions (see future directions).

Chapter 3 focuses on expanding the existing repertoire of genetic tools to study behaviors using larval zebrafish. Several studies have successfully applied optogenetic tools to manipulate behavior in larval zebrafish (Douglass et al., 2008; Arrenberg et al., 2009; Wyart et al., 2009); however, for behaviors like sleep/wake or visual response, a light stimulus may be inherently confounding (Zhu et al., 2009). To avoid the use of light, we wanted to test and adapt other pharmaco- and thermo- genetic tools to manipulate neuronal activity in larval zebrafish. In addition to the three TRP channels discussed in Chapter 3, I have also briefly tested Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) developed by Bryan Roth's group (Alexander et al., 2009) and Ligand Gated Ion Channels (LGICs) pioneered by Scott Sternson's group (Magnus et al., 2011), by expressing them in the trigeminal sensory and Rohon-Beard neurons. However, none of these channels induced a behavior change similar to what was expected from activation of these neurons. In Chapter 3, I show that rat TRPV1, rat TRPM8, and rattlesnake TRPA1 can be used to activate neurons in larval zebrafish using their respective agonists. In addition, high Csn level can induce rapid ablation of TRPV1

expressing neurons. Other members in our lab have also successfully used TRPV1 to activate and ablate neurons in the lateral hypothalamus and dorsal raphe (unpublished data), suggesting that these channels successfully manipulate neuronal activity in deep brain structures in larval zebrafish. The characterization of the TRP channels offers a complementary set of genetic tools that can be used in combination with each other or with other genetic tools for studying behavior in larval zebrafish.

Future directions

Chapter 2 of this dissertation demonstrates that Prok2 can modulate sleep/wake behavior based on lighting conditions, and loss of Prok2 affects daytime activity and sleep levels. Further studies are needed to elucidate the mechanism by which Prok2 signaling regulates sleep/wake behavior and the function of *prok2*-expressing neurons.

The Prok2 overexpression phenotype persists in many known sleep regulator mutants (see Table 2.1). However, peptide overexpression may induce responses outside of the normal physiological range in animals. It may be more relevant to examine the sleep/wake behavior of *prok2-/-* larvae in these sleep regulator mutants. Another informative but brute force method of searching for the downstream regulator of Prok2 signaling is to measure global changes in the transcriptome by RNA-seq analysis in Prok2 overexpression or loss of function mutants. It may be necessary to analyze transcript levels across multiple points in different lighting conditions. In addition, because activation of Prok receptors leads to activation of MAPK signaling pathway *in vitro* (Lin et al., 2002), measuring changes in protein phosphorylation upon Prok2 overexpression or in *prok2-/-* mutants with proteomics analysis may be useful. Together,

these analyses may provide additional insights and identify new sleep regulatory regions to advance our understanding of sleep regulatory mechanisms.

Based on our Prok2 gain and loss of function observations, we expect that activating *prok2*-expression neurons would suppress activity and promote sleep during the day, and increase activity and reduce sleep at night. In contrast, ablating or silencing *prok2*-expressing neurons would have the opposite effects on sleep/wake behavior. To test this hypothesis, we could utilize the TRP channels characterized in Chapter 3 to activate or ablate *prok2*-expressing neurons using a Prok2-specific enhancer. Other available genetic tools such as red shifted channelrhodopsin and enhance nitroreductase could be used as alternative methods for manipulating *prok2*-expressing neurons (Lin et al., 2013; Mathias et al., 2014). To look at the downstream target of *prok2*-expressing neurons, we could take advantage of the transparency of larval zebrafish and monitor global changes in neuronal activity *in vivo* (Ahrens et al., 2013). We could activate *prok2*-expressing neurons by using one of above listed genetic tools and observe changes in GCaMP signal in the entire brain with light sheet microscopy. One particular region of interest is the LC, which is a known arousal center and expresses Prokr2 in larval zebrafish. Although Prok2 overexpression phenotype persisted in *dbh-/-* larvae, it is possible that the neurons of the LC, not just norepinephrine, are responsible for mediating Prok2 signaling.

The availability of genetic tools could also be applied to combinatorial manipulation of neuronal populations in the brain. It would be interesting to look at the interaction between Prok2 with other proposed circadian output factors including TGF-α, CLC and AVP. Because all these peptides reduce locomotor activity in animals, they may

exhibit redundant function in transmitting the central circadian rhythmicity (Kramer et al., 2001; Cheng et al., 2002; Kraves and Weitz, 2006; Li et al., 2009a). Simultaneous genetic silencing and activation of combinations of neurons expressing different circadian output factors may help to better characterize their distinctive functions.

In summary, the work in this dissertation deepens our understanding of sleep regulation and establishes larval zebrafish as an alternative and appropriate model for studying circadian regulation of sleep in humans. With the expansion of existing genetic tools, future studies of circadian regulation of sleep and other behaviors in larval zebrafish may yield more insights about output from the master biological clock in humans.

APPENDIX

GENERATION OF PROK2 AND PROK RECEPTOR MUTANTS

A powerful approach to determine the function of Prok2 is to mutate the corresponding genomic sequence to render it useless and analyze the mutant animals for behavioral changes. This section describes the generation of Prok2 and Prok receptor mutants used in Chapter 2 of the thesis. Two different genome editing methods were used and their efficacy in inducing somatic and germline mutation was compared. These data contributed to a larger dataset generated and published by our lab (Chen et al., 2013).

Zinc Finger Nucleases (ZFNs)

The first method we used to induce targeted mutation in the zebrafish is context-dependent assembly (CoDA) generated ZFNs (Sander et al., 2011a). ZFNs can be designed to bind specific DNA sequences and induce double-strand DNA breaks (Urnov et al., 2010). Repair of ZFN-induced lesion by nonhomologous end joining often leads to deletion and insertion mutation at the site of lesion, creating frameshift mutation and premature stop codons in the targeted gene. Engineering ZFNs used to require either laborious selection process in bacteria or yeast system (Meng et al., 2008; Maeder et al., 2009) or the use of preselected zinc finger modules which reports to have a low success rate (Ramirez et al., 2008; Urnov et al., 2010). CoDA, a publicly available platform, emerged as an alternative method and can generate ZFNs with high success rate and requires only standard cloning techniques (Sander et al., 2011a). Using ZiFIT Targeter (http://zifit.partners.org/ZiFiT), we designed CoDA generated ZFNs targeting Prok2 and both of the Prok receptors (Table A.1).

Gene	Ensemble ID	Site Position	Left Target	Right Target	Spacer
Prok2	ENSDARG00000091616	252-277	CGTACGTAG	GCTGATGGC	ACTACA
Prokr1	ENSDARG00000074182	793-818	TCGCAGATG	GGCGCAGAA	GACGGC
Prokr2	ENSDARG00000090315	854-878	GTGCCGAAT	TCTGCTGGG	TATCC

Table A.1. ZFN target sites for Prok2 and Prok receptors.

DNA fragments encoding zinc finger arrays were synthesized (Epoch Life Science, Inc) and cloned into FokI EL/KK heterodimeric expression vectors as previously described (Maeder et al., 2009).

Transcription Activator-Like Effector Nucleases (TALENs)

Because the DNA sequences that can be targeted using CoDA are limited (Sander et al., 2011a), we used TALENs as a second method for targeted mutagenesis in zebrafish. (Bogdanove and Voytas, 2011; Huang et al., 2011; Sander et al., 2011b). TALENs contain a variable number of 33-35 amino acid repeats, each of which preferentially binds to a specific nucleotide, and can be designed to target any DNA sequences. Two TALEN target sites for Prok2 and both Prok receptors were designed using the ZiFIT Targeter (http://zifit.partners.org/ZiFiT) based on published guidelines for optimal TALEN target sites (Cermak et al., 2011) (Table A.2).

Gene	Ensemble ID	Site Position	Left Target	Right Target	Spacer
Prok2	ENSDARG00000091616	102-151	TGGCATGTG TTGTGCAGT	AGTCTCCG AATGTGCA	CAGTCTGT GGATCCGC
Prok2	ENSDARG00000091616	7-55	TCCAACATT TCTTTCTT	GTGTCGCG TGGTTGGA	TCTCTGCC TGCTGCTT
Prokr1	ENSDARG00000074182	391-439	TACTTGAGG ACTGTGTC	AATGCTCT GCTGGCCA	TCTCTACG TGTCCACC
Prokr1	ENSDARG00000074182	73-124	TATGACATC CCTGTGGAT	ACGAAATTC CCGACACGA	TACGAGGT CCCGGTGG
Prokr2	ENSDARG00000090315	-16-36	TCTCACAGA AACAGCCAT	AGCCACGTG GCAGCTGTA	GCAGGACG CCAATATC
Prokr2	ENSDARG00000090315	94-146	TACGACATG ATGGACTAC	GATGCCGGA CACCACGCA	GGGGTCCCG GCGGAGGA

Table A.2. TALEN target sites for Prok2 and Prok receptors.

TALE repeat arrays were constructed using the REAL Assembly TALEN kit and cloned into the wild type FokI expression vectors as previously described (Reyon et al., 2012).

TALENs are significantly more mutagenic than ZFNs in zebrafish

Targeted mutagenesis in zebrafish using customized ZFNs and TALENs were performed as previously described (Foley et al., 2009). Briefly, ZFN and TALEN expression plasmids were linearized and used as template to generate mRNA followed by polyA tailing. Approximately 50-100 pg of each ZFN or TALEN mRNA pair was injected into the cell of zebrafish embryos at 1 cell stage. mRNA concentrations that were sufficient to cause developmental defects in 10-50% of injected embryos were used to analyze somatic mutation rate and generate germline mutants.

Detection of somatic and germline mutation has been described in detail (Chen et al., 2013). For somatic mutation, genomic DNA from 12 injected embryos for each ZFN and TALEN pair were prepared and amplified with PCR. The PCR products were then processed for deep sequencing with Illumina HiSeq2000. Indels were detected using SHRiMP2 software (David et al., 2011) and BLAT. For isolating germline mutants, zebrafish embryos injected with a ZFN or TALEN pair were raised to adulthood and mated to wild type fish. Genomic DNA was isolated from a pool of 1-6 embryos and targeted genomic regions were amplified using PCR with a reverse primer fluorescently labeled with 6-FAM. Fluorescent PCR products were run on an ABI 3730 DNA analyzer (Applied Biosystems) and analyzed using Peak Scanner (Applied Boisystems) to detect indels and identify injected F0 fish that carries germline mutation.

We found that although both ZFNs and TALENs induced somatic mutation *in vivo*, TALENs induced somatic indels at much greater rate than ZFNs (Table A.3). The average somatic indel rate induced by tested ZFNs and TALENs were 1.21% and 8.90%, respectively. Furthermore, we found that TALENs can generate germline mutation much more efficiently than ZFNs. 37% of TALEN injected F0 fish contained germline mutation, but we failed to find any ZFN injected F0 fish contained germline mutation. Note that we did not characterize the germline mutation rate for Prok2 and Prokr1 targeted by ZFNs because the somatic indel rate was much lower than that induced by TALENs.

Gene	Ensemble ID	Somatic Indel Rate (%)			Germline Mutation Rate (%)	
		TALEN 1	TALEN 2	ZFN 1	TALEN1	ZFN
Prok2	ENSDARG00000091616	5.92	1.47	0.02	31	N/A
Prokr1	ENSDARG00000074182	19.04	0.16	3.48	30	N/A
Prokr2	ENSDARG00000090315	25.50	1.26	0.13	50	0.00

Table A.3. Summary of somatic indel rate and germline mutation rate induced by ZFNs and TALENs. Somatic indel rate indicates the percentage of sequenced reads that contained mutation. Germline Mutation Rate indicates the percentage of injected F0 fish that contain mutations in their germline. N/A indicates no germline mutation rate was not characterized due to low somatic indel rate.

In summary, this work demonstrates that TALENs are much more mutagenic than ZFNs in zebrafish. The observation is consistent with the larger dataset that our lab has generated after testing 84 ZFN pairs and 34 TALEN pairs. A detailed and statistically significant analysis of the efficacy for both genome editing methods can be found in the listed reference (Chen et al., 2013).

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