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Rest in The Zebrafish

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The purpose and function of sleep has been the topic of discussion for several centuries. Though our understanding of the mechanisms underlying the propagation and maintenance of rest states has undergone significant improvement, much remains to be learned with regards to the effects of disrupted sleep on diseased states. A deeper understanding of the neural circuitry and associated phenotypes would allow for the identification of sleep-related pathologies as well as the development of therapies for individuals with sleep disorders. To this end, the zebrafish (*Danio rerio*) pose a great advantage. Zebrafish are a diurnal animal, and the sleep rhythms of this animal are sensitive to environmental changes and pharmacological interventions. In this review, we highlight the common methods of sleep disruption, and discuss some of the effects of sleep disruption in the zebrafish. Disrupted sleep rhythms in the adult animal are linked to changes in gene and protein expression, while behavioral measures of anxiety have produced mixed results. We propose that this variation is a result of the type of sleep disruption as well as the type of anxiety test employed. This beckons the need for further study of the effects of environmental and pharmacological manipulations on the sleep rhythms of the animal. Further, researchers must not rely solely on one test as a measure of stress or anxiety as it provides only a one-dimensional insight.

Sleep is a highly conserved and essential behavior in the animal kingdom. Aside from humans, this behavior has been observed and studied in rodents, non-human primates, cetaceans, birds and in some cases, insects (Siegel, 2008). Across the animal kingdom, sleep is generally regarded as a state of reversible immobility in which consciousness is suspended and response to environmental stimuli is greatly dampened (Campbell & Tobler, 1984). Four criteria outline the behavioral markers of sleep: (1) the assumption of a species-specific posture, (2) behavioral quiescence, or inactivity, (3) an elevated arousal threshold, and (4) state reversibility with stimulation (Campbell & Tobler, 1984). When possible, monitoring methods to record brain activity, such as electroencephalographs (EEGs) allow for the integration of behavioral and physiological markers, providing a more comprehensive definition of sleep.

In humans, sleep may be divided into 4 stages, which fall under two broad categories: rapid eye movement (REM) and non-rapid eye movement (NREM) sleep. Each stage of sleep has a unique electroencephalographic (EEG) profile and associated benefits, including the integration of sensory information and memory consolidation (Gais, Mölle, Helms, & Born, 2002; Tamminen, Payne, Stickgold, Wamsley, & Gaskell, 2010). Several brain areas are involved in the onset and maintenance of sleep and arousal. The neuromodulatory systems that promote arousal are largely excitatory in nature and include hypocretins (also referred to as orexins), noradrenaline, acetylcholine, dopamine and serotonin circuits (Chiu & Prober, 2013). Sleep promoting systems by nature inhibit or dampen systems that underlie wakefulness; modulators include gamma amino-butyric acid (GABA) and galanin (Chiu & Prober, 2013; Saper, Scammel, & Lu, 2005; Steiger, 2007). The systems involved in onset and maintenance of sleep are largely under the control of the suprachiasmatic nucleus (SCN).

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Regulatory Elements and Sleep Disruption

Seated in the lateral hypothalamus, the SCN functions as the circadian clock by maintaining homeostatic rhythms (e.g., temperature regulation, energy metabolism, and arousal) throughout the day. Several proteins have been identified in the regulation of mammalian circadian rhythms: PERIOD1, PERIOD2, PERIOD3, CLOCK, BMAL1, CRYPTOCHROME1 (CRY1), CRYPTOCHROME2 (CRY2), CASEIN KINASE I-DELTA (CSNK1D) and CASEIN KINASE I-EPSILON (CSNK1E) (Albrecht, Sun, Eichele, & Lee, 1997; Griffin , Staknis, & Weitz, 1999; Honma et al., 1998; King et al., 1997; Ko & Takahashi, 2006; Pando & Sassone-Corsi, 2002; Zylka, Shearman, Weaver, & Reppert, 1998). Dimerization of these proteins drives the rhythmic transcription and translation of regulatory feedback loops.

Despite being internally regulated, the SCN remains susceptible to the influences of environmental lighting and drug administration (Zietzer, Dijk, Kronauer, Brown, & Czeisler, 2000). For instance, the disruption of GABAergic transmission prevents the propagation of sleep (resulting in insomnia; Mohler, 2006), while the disruption of hypocretin pathways leave the individual unable to consolidate sleep bouts (resulting in narcolepsy; Nishino, Ripley, Overeem, Lammers, & Mignot, 2000). Administration of antagonists to sleep promoting networks is also effective in preventing sleep. Adenosine is the natural by-product of metabolism and is produced from the consumption of adenosine triphosphate (ATP) and cyclic adenosine monophosphate (cAMP) in the brain (Bjorness & Greene, 2009). The accumulation of adenosine molecules has been proposed to facilitate the homeostatic drive for sleep (Basheer, Strecker, Thakkar, & McCarley, 2004; Boehmler et al., 2009; Porkka-Heiskanen, Alanko, Kalinchuk, & Stenberg, 2002). Caffeine, an adenosine receptor antagonist, is particularly effective in maintaining arousal states by blocking the binding of adenosine to A1R and A2aR receptors within the basal forebrain (Bjorness & Greene, 2009). This prevents the progression of the sleep-promoting homeostatic mechanisms, allowing the individual to remain alert for an extended period of time (Saper et al., 2005; Zietzer et al., 2000).

Acute and extended sleep loss in humans has been linked to impaired cognitive functions and worsened physical states (Drummond & Brown, 2001; Jung et al., 2011). Restricting sleep time to 4 h/night in human adults decreases the parasympathetic tone, while increasing blood pressure and evening cortisol levels (McEwen, 2006), and the acute deprivation of sleep (36 h), equivalent to an individual forgoing one night's rest, results in elevated self-reports of anxiety (Sagaspe et al., 2006). Persistent sleep disturbances (i.e., insomnia and excessive sleepiness) are accompanied by depression, cognitive changes and anxious states (APA, 2013 Mohler, 2006; Riemann, 2007). While there is a link between sleep disruption and anxious states, the exact relationship between the two states remains largely unexplored. In such cases, animal models can provide greater insight to the neural underpinnings that underlie these mechanisms.

For decades, rats and mice have been used in the study of sleep and sleep-associated disorders (Everson, Bergmann, & Rechtschaffen, 1989; Pires, Tufik, & Andersen, 2015; Rechtschaffen, Bergmann, & Everson, 2002). Sleep in rodents is polyphasic. Under typical lab conditions the rat will enter roughly 123 sleep bouts, with the majority of sleep taking place during the day (van Twyer, 1969). The effects of partial and total sleep deprivation are much more pronounced in rodents, with animals dying after 15 days of sleep deprivation on average (Everson et al., 1989). Death is preceded by weight loss, immune system dysfunction, and impaired heat retention mechanisms (Everson, 1993, 1995; Everson et al., 1989; Rechtschaffen et al., 2002).

There has been a recent shift in neurobehavioral psychology from the use of rodent models to that of the zebrafish. While the rodent model has provided a wealth of knowledge and a greater understanding of many

facets of the human condition (e.g., rest, anxiety, disease states), it does have a few drawbacks (e.g., long generation time, small litter size, costly), which make this animal model unsuitable for large scale behavioral screening. Zebrafish provide a unique combination of complexity and simplicity, resulting in easier variable manipulation, faster data collection, and outcomes which are translatable across other animal models.

Sleep and Rest in the Zebrafish

The zebrafish has gained tremendous popularity in neurobehavioral studies, partly due to high genetic homology and phenotypic similarity to rodents and humans (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Howe et al., 2013). This homology is extended to neuro-signaling pathways (e.g., dopamine, serotonin, hypocretin) and brain patterning that makes this model suitable for the study of several behaviors and correlates of human disease states (Dodd, Curtis, Williams, & Love, 2000). Within the past 15 years, the systematic study of rest and arousal in zebrafish has surged. As a diurnal animal, zebrafish exhibit circadian rhythmicity, and light-dependent expression of circadian oscillators (Ben-Moshe, Foulkes, & Gothilf, 2014).

Circadian rhythms begin to develop within the first day post fertilization (dpf), and are fully functional within 4-5 dpf (Ben-Moshe et al., 2014; Hirayama, Kaneko, Cardone, Cahill, & Sassone-Corsi, 2005; Hurd & Cahill, 2002). Zebrafish express several orthologues of the mammalian circadian clock, including Period (Per1a, Per1b, Per2, and Per3), bmal (bmal1, bmal2) and Cryptochrome genes (Pando & Sassone-Corsi, 2002; Ziv, Levkovitz, Toyama, Falcon, & Gothilf, 2005). These clock genes are responsible for regulating growth, locomotion, hormone (e.g., melatonin) production and rest (Danilova, Krupnik, Sugden, & Zhdanova, 2004; Pando & Sassone-Corsi, 2002; Vatine, Vallone, Gothilf, & Foulkes, 2011). Some of the earliest rhythms to emerge are those of exo-rhodopsin (exorh), melatonin producing enzymes (e.g., aanat2) and period genes. Exorh, along with environmental cues and other circadian elements, regulates the production of aanat2, and is responsible for the entrainment of circadian rhythms to environmental cues (Vuilleumier et al., 2005; Ziv & Gothilf, 2006; Ziv et al., 2005). It is hypothesized Per2 acts as the intermediary regulating element between exorh and the transcription of aanat2 (Appelbaum, Anzulovich, Baler, & Gothilf, 2005; Appelbaum et al., 2006). Thus under typical conditions, the production of melatonin in the larval zebrafish begins as early as 2 dpf and is triggered by exposure to light (Ben-Moshe et al., 2014; Kazimi & Cahill, 1999; Vuilleumier et al., 2005). During early development, melatonin plays a crucial role in maintaining circadian rhythms, specifically in cell proliferation and the onset / maintenance of sleep (Danilova et al., 2004; Gandhi, Mosser, Oikonomou, & Prober, 2015).

In cases where the use of EEG, EMG and other physiological measures of sleep cannot be used, several behavioral criteria may be used to classify this behavior (Campbell & Tobler, 1984). These include: a state of rest (quiescence) regulated by circadian rhythms, the assumption of a species-specific posture, reduced sensory responsiveness, and state reversibility with stimulation (Campbell & Tobler, 1984). All four criteria have been met and defined in the zebrafish – though like many species in the animal kingdom, the definitions for sleep are slightly different in the young versus adult animal (Chiu & Prober, 2013; Elbaz, Foulkes, Gothilf, & Appelbaum, 2013). In zebrafish, sleep is defined as a state of immobility in which the animal assumes a species specific immobile posture and exhibits an increase in arousal threshold Physiological markers of sleep include a reduction in the breathing and heart rate (Zhdanova, Wang, Leclair, & Danilova, 2001). While asleep, the animal typically remains towards the top of the water column and assumes one of two postures: floating horizontally, or floating with the head angled slightly downwards (Kalueff et al., 2013; Zhdanova, 2006). In the past, there have been minor differences in the definition of sleep between the larval and adult animal, such that sleep in the larval zebrafish required that the animal maintains postural immobility for a period lasting 2-10 min (Rihel, Prober, & Schier, 2010; Zhdanova, 2006). Sleep in the adult zebrafish would require that the

animal remains immobile for a period of at least 6 s (i.e., the 7th s and beyond of an immobile bout is considered to be sleep; Sigurgeirsson et al., 2013; Yokogawa et al., 2007; Zhdanova et al., 2008). Recently however, Sorribes et al. (2013) discovered there to be no differences in the length of sleep bouts across the zebrafish lifespan. As a result, there has been a shift in the definition of sleep in the larval animal, where now some researchers follow the six-second rule (Srdanović et al., 2017). This allows the researcher to capture more of the nuances in sleep structure in all stages of development (Srdanović et al., 2017).

Sleep and waking rhythms are largely under the control of circadian clocks within the pineal organ and retina. However, these behaviors may also be modulated by environmental conditions (e.g., drug administration, or changes in ambient lighting), or a disruption in the circadian rhythm.

Circadian Clocks

As previously noted, circadian rhythms first develop within 1 dpf, with the expression of exorh in embryonic pinealocytes at roughly 18 hours post fertilization (hpf; Vuilleumier et al., 2005). Exorh, along with environmental cues and other circadian elements, is responsible for the entrainment of circadian rhythms to environmental cues (Vuilleumier et al., 2005; Ziv & Gothilf, 2006; Ziv et al., 2005). The production of aanat2 in the pineal organ begins at roughly 22 hpf and circulating aanat2 levels are regulated by several positive and negative feedback loops.

The first positive transcription loop is responsible for enhancing aanat2 expression. The expression of aanat2 is mediated by the interaction of two complexes: the Clock/Bmal heterodimer and the orthodenticle homeobox 5 (Otx5; Appelbaum et al., 2005). The Clock/Bmal heterodimer enhances aanat2 expression through an enhancer box (E-box) downstream of the aanat2 promoter region, while Otx5 promotes transcription through photoreceptor conserved elements (PCE; Appelbaum et al., 2005).

In the negative feedback loop, the cryptochrome and period genes play a central role. Unlike bmal and clock, the cryptochrome and period genes act as negative clock proteins and suppress the activity of the positive clock regulators (Appelbaum et al., 2006). Zebrafish have six orthologues of the mammalian cry gene (Pando & Sassone-Corsi, 2002). These share functional similarities to the human and rodent cry genes in their ability to disrupt Clock/Bmal transcription. Zebrafish have four orthologues of the mammalian period gene: per1a, per1b, per2, and per3 (Appelbaum et al., 2005; Ziv et al., 2005). Under typical lighting conditions, the expression of period2 mRNA peaks during the day, and is virtually undetectable during the night (Cahill, 2002; Kazimi & Cahill, 1999). The onset of environmental lights stimulates period2 mRNA expression, which through E-box represses aanat2 expression, thereby reducing melatonin production (Elbaz et al., 2013; Wang, Zhong, Yingbin, Zhang, & Wang, 2015). Further, zfper2 has the potential to act as an intermediate between exorh on the cell membrane and clock components such as Clock/Bmal on the aanat2 promoter.

Zfper1 and zfper3 play smaller, yet significant, roles in the regulation of the circadian clock. Per3 has a higher mRNA expression during the daytime and likely suppresses exorh transcription indirectly through the regulation of another gene / mechanism that does not require Clock/Bmal.

Outside of the regulatory clock, several regulatory elements have been identified. For instance, Kcna2, a gene encoding voltage-gated potassium channels is an important regulator of sleep across phyla (Cirelli et al., 2005; Srdanović et al., 2017). In zebrafish, the knockdown of kcna2 reduces sleep, similar to the effects observed in flies and mice (Srdanović et al., 2017), providing evidence of a conservation of function.

Neuromedin U (Nmu) is a neuropeptide that is involved in locomotion and arousal (Nakazato et al., 2000). In zebrafish, the overexpression of Nmu promotes hyperactivity and arousal, whereas nmu knockdown produces arousal defects (i.e., reduction in daytime activity; Chiu et al., 2016). This neuropeptide, though not directly involved in the circadian clock, promotes hyperlocomotion through interaction with the brainstem (Chiu et al., 2016).

Regulating Rest and Arousal Via Pharmacological Intervention and Environmental Manipulation

The experimental manipulation of environmental lights (e.g., constant darkness or constant lights) alters the transcription of certain clock genes, including per2, clock, bmal1 and bmal2, ultimately affecting the circadian rhythms of rest and arousal (Ben-Moshe et al., 2014; Cahill, 2002). In addition to suppressing the expression of clock and bmal genes, an exposure to bright lights causes a further reduction in aanat2 expression, resulting in low levels of circulating melatonin during the day (Árnason, Þorsteinsson, & Karlsson, 2015; Ben-Moshe et al., 2014; Falcon, Miguad, Munoz-Cueto, & Carrillo, 2010; Sigurgeirsson et al., 2013). As a result, activity levels in zebrafish peak during the subjective day, while inactivity and rest behaviors peak during the subjective night (Sigurgeirsson et al., 2013; Zhdanova, 2006, 2011).

In addition to ambient lighting, rest and arousal are susceptible to genetic and pharmacological intervention. The conservation of several neurotransmitter and neuropeptide pathways is well-documented in the zebrafish (Elbaz et al., 2013; Faraco et al., 2006; Prober, Rihel, Onah, Sung, & Schier, 2006; Zhdanova, 2011). Further, the administration of agonists and antagonists of mammalian sleep-regulating mechanisms have comparable effects in the zebrafish, highlighting their potential as a model for studying sleep and related disorders (Rihel, et al., 2010; Rihel & Schier, 2013; Singh, Subhashini, Sharma, & Mallick, 2013; Zhdanova et al., 2008).

Ethanol. Ethanol exposure yields many dose-dependent phenotypes in zebrafish. The acute administration of low doses of alcohol (e.g., 0.5-1% v/v in adult zebrafish, for 20 min) yield anxiolytic responses in the animal and result in an overall increased swim speed and locomotion (Gerlai, Lahav, Guo, & Rosenthal, 2000; Mathur & Guo, 2011). After an exposure to 1% ethanol for 60 min, the mean swim speed is statistically significantly lower, and the animal demonstrates hypolocomotion (Gerlai et al., 2000; Rosemberg et al., 2012; Tran & Gerlai, 2013). This response indicates a dose-dependent effect of sedation in the animal, similar to that observed in humans and rodents (Hendler, Ramchandani, Gilman, & Hommer, 2013).

Ethanol administration produces a neurochemical changes within the brain in a dose-dependent manner, which may account for the observed changes in behavior and locomotion (Tran, Chatterjee, & Gerlai, 2015). Acute ethanol exposure induces an increase in serotonin, dopamine and their respective metabolites 5HIAA and DOPAC in the zebrafish brain (Tran et al., 2015). It has been suggested that ethanol administration increases dopamine and DOPAC levels, by altering levels of tyrosine hydroxylase, the rate limiting enzyme for catecholamine synthesis (Tran et al., 2015). It has been suggested that the increase in serotonin and metabolites are also resultant of an ethanol induced mechanism (Tran et al., 2015).

GABA. GABA is an inhibitory neurotransmitter, and is a prominent modulator of sleep and arousal regulatory systems (Saper et al., 2005). GABA-ergic neurons in the ventrolateral preoptic nucleus (VLPO), via inhibitory action on the hypothalamus and brain stem areas, suppress arousal (Saper et al., 2005). Lesions to the lateral hypothalamus or malfunctioning GABA receptors within the lateral hypothalamus, prevent the VLPO from exerting its inhibitory effect on the circadian rhythms. This leaves the individual vulnerable to insomnia

(Mohler, 2006). The zebrafish GABA-ergic system is well developed (Doldan, Prego, Holmquist, & de Miguel, 1999), and sensitive to pharmacological intervention (Zhdanova et al., 2001). The administration of agonists to the GABA-ergic system has a dose-dependent sedative effect in adult zebrafish (Zhdanova et al., 2001). At low doses, the zebrafish experiences an increase in arousal threshold and a decrease in locomotor activity. In contrast acute exposure to high doses of barbiturates and benzodiazepines have an anesthetic effect, while a prolonged exposure may result in the death of the animal (Zhdanova, 2011).

Histamine. The mammalian histaminergic system is heavily involved in a variety of physiological functions (Sundvik et al., 2011), and functions as an arousal promoting factor (Moree et al., 2009). However, the exact sites of histamine innervation in vertebrates is not well understood (Sundvik et al., 2011). The zebrafish histaminergic system is functionally and developmentally linked with the hypocretin system in the zebrafish (Sundvik et al., 2011). The inactivation of histaminergic systems greatly impact the developing hypocretin system (Sundvik et al., 2011). Histamine antagonists act as sedatives in humans and rodents (Passani, Lin, Hancock, Crochet, & Blandina, 2004). A similar effect is observed in larval and adult zebrafish, with low doses of histamine antagonists (e.g., mepyramine) producing a mild sedation, and high doses producing an anesthetic effect (Renier et al., 2007; Zhdanova, 2011).

Serotonin. The zebrafish serotonergic system begins to develop within 1 dpf, and is fully functional within 5 dpf (Brustein, Chong, Holmqvist, & Drapeau, 2003). Serotonin is involved in organizing locomotor pattern in zebrafish. Exogenous administration of 5-HT decreases the period of quiescence between bouts of swimming activity in the developing embryos, (Brustein et al., 2003), but it reduces locomotor frequency in the adult zebrafish, likely by delaying onset of the excitatory phase in spinal cord neurons (Gabriel et al., 2009).

In developing embryos the administration of exogenous serotonin (5-HT), or the injection of a broad acting serotonin receptor 2/3 agonist, quipazine, significantly increase the frequency of spontaneous swim bouts (Brustein et al., 2003; Rihel, et al., 2010). However, it has been demonstrated that several serotonergic modulating drugs have a high affinity for non-serotonin receptors (Rihel, et al., 2010). This prevents us from fully understanding whether the serotonergic drugs impose their effect via serotonin family receptors, or whether the effect is mediated by other pathways, e.g., via dopaminergic or adrenergic receptors (Rihel, et al., 2010). The chronic administration of agonists (e.g., BMY-7378, BP-554, Buspirone, PAPP, RU-24969) and antagonists (e.g., MM-77, NAN-190, P-MPPF) to serotonin receptor 1A increase rest, a similar effect to that seen in mammals (Rihel, Prober, Arvanites, et al., 2010). Administration of SSRIs such as Fluvoxamine also reduces waking activity in the zebrafish (Rihel, et al., 2010). Antagonists of the serotonin 2/3 receptor have similar paradoxical effects in mammals and zebrafish. In both cases, 5HT¬2/3R antagonists increased total rest time, and waking exploratory behavior (Rihel, et al., 2010).

Melatonin. Melatonin serves many functions in vertebrate animals, ranging from appetite and reproduction regulation to cell proliferation and sleep rhythm maintenance (Lima-Cabello et al., 2014). In the zebrafish, melatonin production begins roughly 18 hpf, and peaks at 24 hpf. This timeline coincides with the appearance of melatonin receptor mRNA (Danilova et al., 2004). Zebrafish express five orthologues of the mammalian melatonin receptors, spanning three distinct subtypes that have been identified in other vertebrates (Reppert, Weaver, Cassone, Godson, & Kolakowski, 1995). Presently, however, the localization of these receptors is unknown (Lima-Cabello et al., 2014).

Under typical laboratory conditions (i.e., 14h L: 10h D), melatonin production is at its peak during the dark phase and is responsible for cell proliferation and rest in this animal (Cahill, 1996, 2002; Danilova et al., 2004; Zhdanova, 2011). The administration of exogenous melatonin induces rest in larval zebrafish,

characterized by a decrease in locomotion and an increased arousal threshold (Zhdanova et al., 2001). Exogenous melatonin induces rest via melatonin receptors, though it is presently unknown which specific receptors mediate the effect (Zhdanova et al., 2001). This effect is observed irrespective of the time of day. The manipulation of endogenous melatonin (achieved by creating null variants of enzymes catalyzing the production of melatonin) also disrupts nighttime sleep rhythms in the animal (Gandhi et al., 2015).

The production of melatonin in zebrafish is regulated by the expression of aralkylamine-N-acetyltransferase (aanat; Cahill, 2002). This is the penultimate enzyme in melatonin production, and is primarily expressed during dark phases (Cahill, 2002). Zebrafish have two orthologues of this gene, aanat1 and aanat2, expressed predominately in the retina and pineal gland, respectively (Appelbaum et al., 2006). Similar to humans, the expression of aanat2 mRNA is largely dependent on environmental conditions (i.e., light vs dark) and the expression of circadian elements (e.g., exorh, and per2; Appelbaum et al., 2006). The aanat1 variant is exclusively dependent on phase change; maintaining animals under constant dark conditions eliminates aanat1 rhythms. The development of a null mutant for the aanat1 gene reveals that this gene does not play a significant role in maintaining sleep rhythms (Gandhi et al., 2015). The introduction of an aanat2 null mutant significantly increases the latency to sleep, and reduces the amount of total sleep time in the animal during the night phase (Gandhi et al., 2015).

Hypocretin. The zebrafish genome possesses a single orthologue for the mammalian hypocretin (HCRT) receptor (Prober et al., 2006). In humans, hypocretin plays a role in maintaining wakeful states, where the disruption of hypocretin pathways or the administration of antagonists to this system render the individual unable to consolidate sleep. Similar effects are observed in the zebrafish. There are roughly 40-45 hypocretin neurons located throughout the zebrafish brain, localized to the posterior hypothalamus (Prober et al., 2006). These neurons project to their receptors along the spinal cord and in the telencephalon, diencephalon and hindbrain, and modulate the rest-arousal transitions in the animal (Appelbaum et al., 2009; Faraco et al., 2006; Prober et al., 2006; Sundvik & Panula, 2015; Yokogawa et al., 2007). In a non-imaging technique that allows for the monitoring of neural activity in vivo, Naumann, Kampff, Prober, Schier and Engert (2010) created a transgenic cell line with Aequorin, a bioluminescent protein produced by the jellyfish Aequorea Victoria. Upon activation of the Green Fluorescent Protein - Aequorin (GA) complex by calcium, a series of catalyzing reactions is set into motion that ultimately results in the emission of a green photon (see Nauman et al., 2010 for a detailed report of the methodology). Researchers are capable of targeting GA to specific neurons by expressing the GA complex under the control of the respective promoters. Expressing GA under the control of an HCRT promoter specifically targets the hypocretin neurons in the larval diencephalon. In their study, Naumann et al. (2010) demonstrated that an increase in luminescence is correspondent with an increase in spontaneous locomotion upon arousal in the morning (ZT = 0), providing support for the importance of hypocretin / orexin systems in locomotion and arousal in the zebrafish.

Several researchers have investigated the effects of ectopic hypocretin administration (e.g., injection in to the brain) in non-human animals. Though effective, this technique is invasive, time intensive and produces only transient effects. Transgenic lines produce long lasting effects that allow the researcher to investigate the desired effect at several stages of development. The overexpression of hypocretin gene (hcrt) in larval zebrafish has lasting and drastic effects in the animal. In addition to increasing locomotor activity, hcrt overexpressed larvae experience fewer and shorter sleep bouts (Prober et al., 2006). This is accompanied by a decrease in arousal threshold, meaning the animal is hypersensitive to arousing stimuli (Prober et al., 2006). The reduced ability to initiate and maintain rest states is one of the hallmark symptoms of individuals with chronic insomnia. At the other end of the spectrum, drastically reducing hypocretin, i.e., via ablation of hcrt neurons, increases the total rest time in the animal while leaving the locomotor pattern and activity unaffected (Elbaz, Yelin-

Bekerman, Ncenboim, Vatine, & Appelbaum, 2012). Hypocretin neuron ablated animals also present an increase in the frequency of sleep to wake transitions during both the day and night, similar to the sleep fragmentation observed in human patients with narcolepsy (APA, 2013; Elbaz et al., 2012).

There is strong evidence suggesting that hypocretin circuitry may modulate pineal gland activity. Hypocretin neurons have been found to project to the pineal gland in both, larval and adult animals. As such, this peptide is closely related with melatonin production. The perfusion of an ex-vivo pineal gland at ZT 21-23 (when melatonin production is decreasing) was found to significantly increase the output of melatonin; Appelbaum et al., 2009). The in vivo analysis of the melatonin-hypocretin relationship was made available through the development of an hcrtr null mutant, effectively eliminating HCRT input to the pineal gland. In these null mutant animals, the production of nighttime melatonin decreased by 30%; further these animals were hypersensitive to the effects of exogenous melatonin (Appelbaum et al., 2009).

Norepinephrine. Norepinephrine (NE) functions as a neurotransmitter and a hormone in humans. The primary function of NE is to mobilize the brain and body. As such, NE release is highest when the individual is awake and alert (Dodt, Breckling, Fehm, & Born, 1997; Irwin, Thompson, Miller, Gillin, & Ziegler, 1999). The administration of NE in humans and rodents promotes wakefulness while preventing NREM and REM sleep (Mitchell & Weinshenker, 2010). The pharmacological inhibition of NE via antagonists induces sleep states (Mallick et al., 2002). Similar effects are observed in the zebrafish. While the release pattern of NE in this animal remains untested, the administration of adenosine antagonists (e.g., prazosin) modulates the effect of environmental conditions (Singh et al., 2013). The inhibition of NE via adrenoceptor antagonists increases total sleep during the day and night. Larval animals treated with prazosin will enter sleep states more frequently, and remain asleep for a longer period of time (Singh, Oikonomou & Prober, 2015). This effect continues even under extended light presentations – it has been demonstrated that the presentation of bright lights for an extended period of time (i.e., greater than 14 h) will disrupt circadian rhythms and virtually prevent the onset of sleep in zebrafish (Sigurgeirsson et al, 2013; Singh et al., 2013). Pretreating zebrafish with prazosin prior to an exposure to extended lights increases the amount of time the animals spent in sleeping, without altering the locomotor activity (Singh et al., 2013), suggesting a similar functionality of norepinephrine in this teleost fish.

Norepinephrine production also plays a role in the functionality of other neuropeptides, particularly hypocretin. The locus coeruleus is the major center for NE production in humans and zebrafish. It is also heavily innervated by hypocretin neurons. In mammals, and in zebrafish, hypocretin is proposed to promote wakefulness by stimulating the noradrenergic pathways in the LC (Prober et al., 2006; Singh et al., 2015). Recent work with zebrafish possessing a null mutant for NE receptors [hsb -/-] reveal the importance of NE in hypocretin induced wakeful states. After heat shock at 6dpf, zebrafish that have been genetically modified to overexpress hcrt [Tg(hsp:Hcrt)+/-] will remain in a wakeful state for longer periods of time. If animals from the Tg(hsp:Hcrt)+/- line are crossed with animals with a null mutant for NE receptors, the resulting progeny will overexpress hcrt, and not possess functional NE receptors (Singh et al., 2015). In these animals, total amount of sleep lost before and after heat shock is modulated by the presence of functional NE receptors. Animals that overexpress hypocretin protein (Hcrt), and possess functional NE receptors will undergo greater sleep loss. In contrast, animals that overexpress Hcrt, but do not possess functional NE receptors will lose only a fraction of the total sleep post heat shock. The mediating effect of NE receptors on Hcrt-induced wakefulness highlights the complex interconnectedness of the various neuropeptidic pathways.

Environmental manipulation. In addition to the sensitivity of the sleep-wake systems to HCRT and NE pathway disruption and drug administration, zebrafish remain susceptible to changes in environmental

conditions (Sigurgeirsson et al., 2013). Rest behaviors may be interrupted by several routes in the zebrafish, including extended presentation of bright lights, and administration of electric shocks (Sigurgeirsson et al., 2013). The presentation of bright lights (>200 lux) for an extended period of time (e.g., 24+ hours) effectively reduces the total number of sleep to wake transitions and the total amount of time spent in a sleep state (Sigurgeirsson et al., 2013). The administration of low voltage electric shock (< 6V/cm) to the tank (Sigurgeirsson et al., 2013; Yokogawa et al., 2007) also disrupted adult zebrafish rest, reducing the total sleep time by 30% (Yokogawa et al., 2007). This procedure produces partial habituation in the animal such that the resting zebrafish becomes increasing unresponsive to electrical stimulation. Despite the partial habituation, significant sleep rebound was observed in the sleep deprived zebrafish when released into an extended period of darkness, suggesting that electrical stimulation is an effective method of disrupting sleep in zebrafish (Sigurgeirsson et al., 2013; Yokogawa et al., 2007). Other methods of disrupting sleep have involved mechanical stimulation (e.g., tapping gently on the side of the tank), or acoustic interference (e.g., by placing an underwater speaker in the bottom of a tank). Both methods are initially effective in disrupting sleep. However, the zebrafish demonstrate strong habituation to the stimuli. When sleep is disturbed or prevented via electric shock or constant vibration during the night phase, the animal will demonstrate a heightened arousal threshold, and a reduced activity level after the stimulus is removed (Chiu & Prober, 2013; Yokogawa et al., 2007; Zhdanova et al., 2001). The presence of sleep rebound has not been evaluated following sleep disruption via pharmacological manipulations.

The extended presentation of bright lights, and the administration of low voltage electric shocks are effective tools in the disruption and prevention of sleep, and have been widely used to study the genetic and behavioral effects of sleep deprivation (Purushothaman et al., 2015; Singh et al., 2013; Yokogawa et al., 2007). However, much remains to be learned with respect to the effect of sleep disruption, or environmental manipulation on gene expression and behavior. Further, the majority of the zebrafish sleep deprivation work has been conducted with animals deprived of one night of sleep. It would be interesting to understand the effects of sustained partial or total sleep deprivation in the zebrafish on immunity, anxiety, social behaviors and more.

Effects of Sleep Rhythm Disruption

The disruption of sleep via changing environmental conditions (i.e., extended darkness, extended lights, and electric shocks) affects genomic and proteomic expression as well as sleep profile. A total of 279 gene transcripts are upregulated in zebrafish following an extended light exposure. These are largely related to cell proliferation, plasticity, and energy expenditure (Sigurgeirsson et al., 2013). Twenty-five proteins involved in the maintenance of circadian rhythms are differentially regulated during an extended exposure to light or dark environments. Under bright conditions (normal exposure), there is an up-regulation of cry and clock genes, and a down-regulation of the bmal transcripts (Purushothaman et al., 2015). During extended dark exposure, the per1a, per1b, per3, cry1b, and cry3 transcripts are upregulated, while bmal1b and bmal2 are downregulated. During an extended light exposure per1a, per3, and bmal2 genes are significantly upregulated while cryb is downregulated (Purushothaman et al., 2015). The differential protein expression correlates with the disruption in sleep rhythms observed in adult zebrafish following light-dark cycle manipulation. An extended exposure to dark conditions increases the total amount of time spent in a sleep state during the daylight hours (i.e., 8am – 10pm), as well as the number of sleep-wake transitions as compared to control animals (Sigurgeirsson et al., 2013). Total sleep time is reduced when animals are maintained under extended bright environmental conditions; sleep may also be reduced with the administration of electric shocks or mechanical vibrations (Sigurgeirsson et al., 2013; Zhdanova et al., 2001). As is seen in humans (Drummond

& Brown, 2001; Jung et al., 2011; Leproult, Copinschi, Buxton, & Van Cauter, 1997; Sagaspe et al., 2006) and rodents (Campbell, Guinan, & Horowitz, 2002; Silva et al., 2004; Tartar et al., 2009), the disruption of sleep rhythms has the capacity to elicit anxiety-like behaviors in the zebrafish (Sigurgeirsson et al., 2013; Singh et al., 2013). Recent work has evaluated the anxiogenic effects of sleep rhythm disruption in the zebrafish, producing mixed results.

Behavioral measures of anxiety in zebrafish typically employ one of three tasks: novel tank test, light-dark preference, and open field (Champagne et al., 2010; Egan et al., 2009; Stewart et al., 2012). In each of these tasks, the placement of the animal(s) is recorded along with several other behavioral parameters (e.g., distance traveled, average velocity, erratic swimming and freezing behavior; Egan et al., 2009; Kalueff et al., 2013; Stewart et al., 2012). In the novel tank test, the focal animal is placed into a novel environment following exposure to a stressor. The novel tank test rests on the tendency of the animal to dive to the lower regions of an environment in the face of a stressor, or immediately following the presentation of a stressor; to test vertical swimming, the tank employed in the novel tank test is typically narrow, allowing for swimming mainly in the vertical axis. The tendency for the animal to remain in the lower regions of their environment (a behavior dubbed geotaxis; Kalueff et al., 2013), and a latency to explore the higher regions of the environment are both indicative of anxiety like behaviors in the animal (Champagne et al., 2010; Egan et al., 2009; Stewart et al., 2012). In the light-dark preference task, the focal animal, following exposure to a stressing stimulus, is introduced into the center of a rectangular tank. In the light-dark test, the zebrafish is freely able to swim between two sides of the tank, each of which has a different lighting condition. Typically, the whole tank is under standard illumination (e.g., 150-250 lux); one half of the tank being covered in a white liner, while the other is covered with a black or dark liner, affording an opportunity for hiding. In the light-dark preference test, the zebrafish is allowed to swim freely between the two sides; the tendency to remain in the darker regions (scototaxis) along with differences in distance, velocity, erratic swimming and freezing behavior indicate anxiety like states in the animal (Champagne et al., 2010; Egan et al., 2009; Stewart et al., 2012). The open field task is modeled after the traditional rodent task, and centrally focuses on the animal's tendency to explore the middle regions of the environment as compared to wall-hugging behavior (thigmotaxis; Egan et al., 2009). Thigmotaxic behavior is said to be indicative of a want to escape from the environment; following an exposure to a stressor, animals will exhibit greater thigmotaxic behavior in the open field and demonstrate differences in erratic swimming, freezing, and distance swum/velocity (Champagne et al., 2010; Egan et al., 2009; Stewart et al., 2012). Physiological markers of a stress response may be evaluated through the quantification of heat shock proteins (hsp) or cortisol in the tank water (Félix, Faustino, Cabral, & Oliveira, 2013; Hallare, Pagulayan, Lacdan, Kohler, & Triebskorn, 2005), whole-body cortisol in adult or larval zebrafish (Yeh, Glock, & Ryu, 2013), and vein and trunk cortisol samples (Pavlidis, Sundvik, Chen, & Panula, 2011). A heightened anxiety-like response is typically accompanied by an increase in cortisol and hsp concentrations (Canavello et al., 2011; Hallare et al., 2005).

Disruption of sleep rhythms (e.g. via presentation of lights or electric shock) tends to yield an anxiety-like response in zebrafish and other teleosts (Peter, Hontela, Cook, & Paulencu, 1978). An extended exposure to bright environments (reducing total sleep time) results in increased scototaxis (Singh et al., 2013). While this is suggestive of a wake-induced anxiety-like response, it is unknown if the animals remained in this region of the tank to rest. Previous research has demonstrated sleep rebound in larval and adult zebrafish, although this effect was less pronounced in the adult animal (Chiu & Prober, 2013; Yokogawa et al., 2007; Zhdanova et al., 2001). Disrupting sleep rhythms via the presentation of bright lights or low-voltage electric shocks results in a reduced place preference for top versus bottom in the novel tank test and unaltered whole body cortisol (Sigurgeirsson et al., 2013). Each of the described tasks measures a slightly different swim parameter – the novel tank test measures diving behavior, the light-dark preference test measures hiding tendency, and the

open field task evaluates exploratory behavior. In the face of measuring anxiety following sleep deprivation or disruption, the novel tank test and the open field test would be better suited to measure boldness and exploratory behaviors, relative to the light-dark preference test, as the dark environment could provide an opportunity for rest, thereby giving an inaccurate measure of anxiety like behaviors.

In most circumstances, anxiety-like behaviors are measured in a single behavioral task, providing a one-dimensional insight to the resultant behavior. Because zebrafish are able to produce behaviors in three dimensions, and anxiety-like behaviors are evaluated in tanks of various shapes and sizes, it would behoove researchers to evaluate behavior using more than one test. Further, accompanying behavioral tasks with cortisol measurements could provide further insight to the effects of sleep deprivation on anxiety. In teleosts, cortisol release follows daily circadian fluctuations (Peter et al., 1978). We are not aware of any study to date that has evaluated daily cortisol fluctuations in the zebrafish, however it would follow that a deprivation of rest and a disruption in environmental lighting would result in an alteration of circadian hormone and steroid fluctuations throughout the day.

However, this speculation necessitates additional analysis of typical sleep behavior (during the light phase and the dark phase) to enhance our understanding of the interaction between sleep and anxiety.

Discussion

The study of sleep in zebrafish has gained traction within the past decade. Our understanding of zebrafish sleep structure and function is still in its infancy, but this branch of research is very promising. Studies to date have largely examined the effects of various pharmacological manipulations (e.g., orexin, melatonin, GABA; Zhdanova et al., 2008) and environmental conditions (e.g., prolonged darkness, brightness and electrical stimulation; Sigurgeirsson et al., 2013) on swim activity in adult zebrafish throughout the night. Little is known about the effects of such pharmacological and environmental manipulation at the genetic and proteomic levels, and the effects on the subsequent anxiety profiles are often contradictory.

The zebrafish model presents a unique combination of neural simplicity and behavioral complexity that allows for the translation of behaviors to rodents and humans. With its short generation time and ease of genetic and pharmacological manipulation, the zebrafish model offers researchers many opportunities to study the confluence of genetics and the environment. Previous work has demonstrated the similarity in sleep structure of the zebrafish to humans, highlighting the conservation of sleep and wake regulating circuits (Sorribes et al., 2013). However, the effects of sleep disruption on factors such as anxiety or cognition has received very little attention, and in many cases, the observed effects vary. This may be due to the type of task employed, and even the strain of zebrafish being tested as different strains have been shown to elicit different behavioral responses, namely in tasks of aggression and exploration (Vignet et al., 2013). Using more than one type of task to evaluate behavior allows the researcher to understand the effect of a pharmacological or genetic manipulation in different environments, which may elicit different types of behaviors, and shed light on the behavioral effects of the manipulation (Khan et al., 2017). For instance, in the study of sleep deprivation on anxiety, the researcher may choose to evaluate behavior in the novel tank test and the open field – two tasks which allow for vertical and horizontal exploration, respectively.

The use of zebrafish as a neurobehavioral model is well established, and has provided great insight in the fields of cognition, addiction and memory (Bilotta, Barnett, Hancock, & Saszik, 2004; Egan et al., 2009; Gerlai, 2012). Their utility as a model of sleep and rest behaviors has also received much attention in the recent

years (Sigurgeirsson et al., 2014; Sorribes et al., 2013; Zhdanova, 2006). The high genetic homology allows for the translation of genotypic studies to higher order mammals such as rodents and humans. While the zebrafish cannot replace rodents as a translational model, they certainly complement the existing wealth of knowledge. The zebrafish model also allows for the study of a wide range of behaviors, which has the potential to inform us about the relationships between the sleep-wake systems and other vital functions, including shoaling behavior and cognition.

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