Chapter 4

Conclusions

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Sleep is a highly conserved behavioral state whose regulation remains unclear. The current model postulates a regulation by a homeostatic and a circadian process, but the mechanisms are not clear. This thesis examines the role of melatonin and adenosine signaling in sleep wake behavior of zebrafish and specifically clarifies the role of endogenous melatonin in the regulation of sleep-wake behavior in vertebrates. The study generates and uses several mutant zebrafish lines for the study of the role of Melatonin and Adenosine in the regulation of sleep. The salient conclusions of this thesis are described briefly below.

Melatonin and zebrafish sleep

Studying the role of endogenous melatonin in sleep behavior, the research describes the first diurnal vertebrate genetic loss of function model for endogenous melatonin. We show that *aanat2* mutant zebrafish larvae take twice as long to fall asleep and sleep only half as much as controls at night in LD conditions. Though, it has been established that exogenous melatonin induces sleep behavior in zebrafish (Zhdanova et. al., 2001) and that endogenous melatonin is regulated by the circadian clock (Kazimi and Cahil 1999) this study provides the first evidence that depletion of endogenous melatonin in zebrafish results in a significant decrease in night-time sleep.

Endogenous Melatonin's role in sleep regulation

Although exogenous melatonin has been shown to be a somnolent in zebrafish and other organisms, the role of endogenous melatonin was unclear. Experiments with endogenous melatonin are difficult to carry out in mice, since many common laboratory mice strains possess mutations, which leave them deficient in melatonin. In addition,

melatonin being a 'dark hormone' is produced at night in nocturnal rodents, so is co-incident with high activity level and low sleep levels for rodents. Zebrafish provide a good alternative in this context. Mutating *aanat2* in zebrafish effectively depletes endogenous melatonin levels as was shown by ELISA. This was shown to decrease sleep levels at night in zebrafish. To further examine the role of endogenous melatonin on sleep behavior, we also perform a selective reversible depletion of melatonin-producing pinealocytes, resulting in a decrease in sleep levels during the night. This method is specific and reversible and effectively targets only melatonin producing cells, in contrast to pinealectomy operations performed in rodents (Fisher and Sugden, 2010; Mendelson and Bergmann, 2001; Mouret et al., 1974). This alternative form of endogenous melatonin depletion also results in a decrease in sleep levels at night in zebrafish.

Zebrafish larvae lacking endogenous melatonin take double the amount of time to fall asleep and spend only half as much time sleeping as controls at night in LD conditions. This effect is surprisingly large since exogenous melatonin has relatively subtle sleep promoting effects in humans compared to prescribed hypnotics (Brzezinski et al., 2005; Buscemi et al., 2006), which has led some to argue that melatonin is not an important sleep regulator (van den Heuvel et al., 2005). However, most hypnotics inhibit neuronal activity throughout the brain by activating GABAA receptors, which is not a physiologically relevant mechanism of sleep promotion (Zhdanova, 2005). Indeed, such an overpowering mechanism of sleep induction would be maladaptive. Further, while exogenous melatonin may be a relatively weak sedative, it does not necessarily follow that endogenous melatonin does not play an important role in sleep. Comparing the importance of endogenous melatonin in humans and zebrafish will require more potent

and specific melatonin receptor antagonists. While melatonin may play a more important role in promoting sleep in zebrafish, our results demonstrate that endogenous melatonin plays a significant role in promoting initiation and maintenance of sleep at night in a diurnal vertebrate.

Melatonin doesn't require a functional circadian clock to affect sleep-wake behavior

It has been proposed that melatonin promotes sleep indirectly by phase-advancing the circadian clock (Arendt, 2003) or inhibiting the circadian drive for wakefulness (Scheer and Czeisler, 2005). If these hypotheses are correct, *aanat2*$^{-/-}$ larvae should have little or no sleep phenotype in the absence of entrained circadian rhythms. We tested this hypothesis by raising larvae in constant darkness, which abolished cellular circadian oscillations, at least in the brain. The *aanat2*$^{-/-}$ sleep phenotype persisted under these conditions, suggesting that endogenous melatonin does not promote sleep by modulating the circadian clock, but rather directly affects the sleep regulatory system.

Behavioral and molecular circadian rhythms do not require melatonin

Exogenous melatonin can entrain the circadian clock in free- running animals (Lockley et al., 2000; Sack et al., 2000) and phase-shift the clock in some contexts (Lewy et al., 1992), we found that endogenous melatonin is not required to initiate or maintain molecular or behavioral circadian rhythms in zebrafish. This observation does not support the hypothesis, based on exogenous melatonin, that endogenous melatonin regulates circadian rhythms (Elbaz et al., 2013). Rather, our data suggest that melatonin acts downstream of the clock to promote sleep.

Melatonin is required for the circadian expression of sleep and mediates process C

A prominent model posits that sleep is regulated by a homeostatic process responding to internal cues for sleep need (process S), and a circadian process responding to external cues (process C) (Borbély, 1982). Evidence in mammals suggests that factors such as adenosine, nitric oxide and prostaglandin D2 play important roles in mediating the homeostatic process (reviewed in Brown et al., 2012). However, while the circadian clock mechanism has been described in detail (Fisher et al., 2013), molecules that convey circadian information to regulate sleep are largely unknown. A factor mediating process C should fulfill three criteria. First, the clock should regulate the level or activity of the factor. Second, administration of the factor should induce sleep during the circadian waking period, but not the sleep period. Third, loss of the factor should abolish circadian regulation of sleep. Peptides whose expression oscillates in a circadian manner, and whose overexpression inhibits activity or promotes sleep during the circadian waking period, have been identified in nocturnal rodents, including cardiotrophin-like cytokine (Kraves and Weitz, 2006), transforming growth factor alpha (Kramer et al., 2001) and prokineticin 2 (Cheng et al., 2002). However, loss-of-function studies have revealed little or no effect on the circadian regulation of activity or sleep (Hu et al., 2007; Kraves and Weitz, 2006; Li et al., 2006; Roberts et al., 2006). Melatonin is an alternative candidate for mediating process C since, similar to these peptides, the circadian clock regulates its production (Klein, 2007) and it can induce sleep in some contexts (Fisher et al., 2013). Indeed, exogenous melatonin potently increases sleep and decreases locomotor activity in zebrafish larvae during the day (Zhdanova et al., 2001), and we find that circadian regulation of sleep is abolished in aanat2$^{-/-}$ larvae. These results suggest that melatonin

mediates process C in the diurnal zebrafish animal model. This discovery may have important implications for the treatment of sleep and circadian rhythm disorders in humans.

Melatonin may regulate sleep by activating adenosine signaling

An open question raised by the two-process model (Borbely, 1982) is how homeostatic and circadian cues are integrated. Similar to melatonin, we found that activating adenosine signaling promotes sleep and inhibits activity during the day, but has no effect on sleep at night in WT (data not shown) and *aanat2*$^{+/-}$ larvae. In contrast, activating adenosine signaling increases sleep and decreases activity at night in *aanat2*$^{-/-}$ larvae to the same level as their *aanat2*$^{+/-}$ siblings. This result is unlikely due to a ceiling effect for sleep or parallel modulation of sleep by melatonin and adenosine because increasing nighttime sleep using a different approach, using a histamine H1R antagonist, increases nighttime sleep for both *aanat2*$^{+/-}$ and *aanat2*$^{-/-}$ larvae to a similar extent. These results suggest the sleep-promoting effect of endogenous melatonin may be mediated, at least in part, by adenosine signaling, and suggest a potential mechanism linking homeostatic and circadian regulation of sleep.

This hypothesis must be further tested using genetics and measurements of adenosine levels, which will be challenging in the zebrafish due to its large number of adenosine receptor genes and its small brain size. Application of genome editing technologies to diurnal melatonin-proficient mammals would allow the use of genetics and measurement of adenosine levels using available technologies (Porkka-Heiskanen et al., 1997; Schmitt et al., 2012).

Adenosine receptor agonists and antagonists affect sleep-wake behavior in zebrafish

Adenosine receptors 1 and 2A (A1 and A2 respectively) are known to play a role in mammalian sleep behavior, on the basis of agonist/antagonist experiments in rodents. It has been previously reported that the zebrafish A1 receptor antagonists as well as general adenosine receptor agonists affect sleep-wake behavior in zebrafish. We firmly establish that the A1 receptor is involved in zebrafish sleep, by showing that an agonist for A1 increases sleep during the day and night as shown in rodents. We also show that a known agonist for the A2A receptor has a similar effect on sleep, i.e. it increases sleep during both the day and night. These results taken together suggest that adenosine signaling plays a role in the regulation of sleep behavior in zebrafish.

The adenosine A1 receptor antagonist activates *pacap* cells in the hindbrain

The adenosine A1 receptor has been reported to be widely expressed in the rat brain with the cerebral cortex, hippocampus, cerebellum, thalamus and brainstem having particularly high expression levels (Reppert et al., 1991; Rivkees, 1995; Weber et al., 1990). A2AR is expressed less widely in the brain than A1R. In order to identify regions of the brain where adenosine signaling mediates its effects on sleep in zebrafish, we performed in-situ staining for the immediate early gene *c-fos* following acute drug treatment. Co-locolization experiments reveal that the A1 antagonist activates a cluster of pacap positive cells in the hind-brain. Other cell populations activated include *gabaergic* populations in the forebrain and hindbrain, *th2, tph1a* and *vmat* positive cell populations in the caudal hypothalamus and *sox2* positive areas in the hindbrain ventricle. Our study reports novel A1 antagonist activated brain sleep-wake regulating areas in zebrafish.

The adenosine A2A receptor agonist activates *vmat* cells in the caudal hypothalamus.

The Adenosine A2A receptor expression has been characterized for rats. Higher expression levels are seen in striatum, nucleus accumbens and olfactory tubercle (Dixon et al., 1996) in rats. Experiments involving the microdialysis or injection of the A2A agonists and antagonists into the rodent brain have revealed the involvement ventro-lateral pre-optic nucleus (VLPO), ventro lateral hypothalamus and tuberomamillary nucleus (TMN) in the effect of A2AR on sleep (Schammel TE 2001, Hong ZY 2005, Satoh 2006, Kumar S 2013). On acute treatment with the A2A agonist CGS21680, activation of a population of cells in the caudal hypothalamus as well as the forebrain was observed. The activated cells in the caudal hypothalamus are also positive for *vmat*. It seems possible that the A1 antagonist and A2A agonist may activate similar or closely situated cell populations in the caudal hypothalamus. This is surprising, considering the behavioral phenotypes of the two drugs is very different. Further identification of these cell populations, should provide more clues as to the identity of the cells mediating the agonist behavioral phenotype. Our study reports novel A2A agonist activated sleep-wake regulating areas of the zebrafish brain.

Adenosine receptor mutants exhibit normal amounts of sleep and activity

There is a discrepancy in the results regarding adenosine receptors wherein for rodent experiments administration of adenosine agonists and antagonists leads to large changes in sleep-wake amounts. However, single receptor mutants do not exhibit any defects in sleep/wake architecture. One possibility is that this is because of developmental compensation in the mutant animals. We tried to preempt any

developmental compensation in the mutants by testing 4dpf zebrafish larvae. We were able to find 5 putative paralogs to the A1 and A2 receptors in zebrafish, 3 for A1R and 2 for A2AR. This multiplicity in genes is probably due to a theorized genome duplication event in the ancestry of zebrafish (Force et al., 1999; Postlethwait et al., 1998; Woods et al., 2000). All of these paralogs show good conservation of residues shown in human adenosine receptors to be important for agonist antagonist binding (Olah and Stiles, 1992, 2000). We generated a double mutant for two A1 receptor paralogs in zebrafish A1a and A1b and the two A2A receptor paralogs, A2Aa and A2Ab, but we could not detect any significant differences in sleep wake architecture between the mutant and wild-type animals. This suggests that either developmental compensation is not the cause of the discrepancy between mutant and drug phenotypes, or there is still developmental compensation in 4dpf larvae. It is possible that change in sleep-wake architechture between mutants and wildtype animals will only be seen under sleep deprivation conditions, when there is an increased homeostatic pressure. It is still to be tested if these zebrafish mutants show defects in recovery sleep following sleep deprivation

Adenosine receptor mutants exhibit normal sensory responsiveness

We would expect, that adenosine receptor mutants would exhibit defective sleep homeostasis machinery, hence would be more aroused by sensory stimuli. To test this hypothesis, we used a mechanical tapping assay. We found that adenosine A1a/A1b double mutant and A2Aa/A2Ab double mutant sensory responses are indistinguishable from their heterozygous sibling controls.

Adenosine receptor 1 agonist phenotype is mediated by the zebrafish A1a receptor

There are three A1 receptor paralogs in zebrafish. All of these paralogs show good

conservation of residues important for agonist antagonist binding in the human A1 (Olah and Stiles, 1992, 2000). However, it is not clear whether all of these paralogs are functional. It is also unknown whether the A1R agonist and antagonist phenotype in zebrafish is mediated by the zebrafish A1 paralogs. Our results indicate that the zebrafish A1a is necessary for the sedation phenotype of the A1 receptor agonist. This establishes a role for the zebrafish A1a receptor in sleep behavior.

Adenosine A1 antagonist and adenosine A2A agonist phenotypes are not mediated by the zebrafish receptor paralogs

We found, unexpectedly, that the mutated zebrafish paralogs are not needed to mediate the effects of the A1 antagonist and A2A agonist. This still leaves the possibility that these drugs work through other adenosine receptors that we have not knocked out yet, which have affinity for the tested drugs.

Summary:

Sleep is an evolutionarily conserved behavioral state whose regulation is poorly understood. A classical model posits that sleep is regulated by homeostatic and circadian mechanisms. Several factors have been implicated in mediating the homeostatic regulation of sleep, but molecules underlying the circadian mechanism are unknown. Here we use animals lacking melatonin due to mutation of arylalkylamine N-acetyltransferase 2 (*aanat2*) to show that melatonin is required for circadian regulation of sleep in zebrafish. Sleep is dramatically reduced at night in *aanat2* mutants maintained in light/dark conditions, and the circadian regulation of sleep is abolished in free-running conditions. We find that melatonin promotes sleep downstream of the circadian clock as

it is not required to initiate or maintain circadian rhythms. Additionally, we provide evidence that melatonin may induce sleep in part by promoting adenosine signaling, thus potentially linking circadian and homeostatic control of sleep.

The brain energy hypothesis of sleep suggests that, sleep is induced when the energy stores of the brain are depleted and low energy molecules such as adenosine accumulate. The adenosine receptors Adora1(A1R) and Adora2(A2R) are known to be involved in this regulation in higher vertebrates. Here we attempt to show that regulation of sleep by adenosine is conserved in zebrafish, and that the zebrafish A1R and A2AR may be involved by testing them with the A1R agonist and antagonist SENBA and DPCPX and the A2A agonist CGS21680. We found that zebrafish possess 3 paralogs of the A1R and 2 paralogs of the A2AR. There is a known discrepancy between the strong effects on sleep wake behavior induced by agonists and antagonists, and absence of any changes in sleep wake architecture seen in mutants. To test whether this is because of developmental compensation, we generated zebrafish Adora1a(A1aR) and Adora1b(A1bR) mutants as well as Adora2Aa(A2AaR) and Adora2Ab(A2AbR) mutants and tested their sleep wake architecture at 5-7 days post fertilization (dpf). We found that they exhibit normal sleep wake patterns, suggesting that developmental compensation doesn't explain this discrepancy. We then show that the antgonists and agonists act on specific brain regions. We discovered that *pacap* cells in the hindbrain, GABAergic cells in the forebrain and hindbrain, dopaminergic and serotonergic cells in the caudal hypothalamus and sox2 positive cells in the hindbrain ventricle are activated by the adenosine receptor 1 antagonist DPCPX. CGS21680, the A2A agonist activates a population of caudal hypothalamic cells positive for *vmat*. This suggests that all these

areas may be involved in adenosine signaling induced sleep-wake behavior. We found that the A1 agonist SENBA requires the zebrafish A1a receptor for its affects on sleep-wake behavior. However, neither A1a nor A1b is sufficient to mediate the role of DPCPX on sleep-wake behavior and the A2Aa and A2Ab are not sufficient to mediate the effects of CGS21680 for its effects on sleep-wake behavior.

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