The Regulation of Sleep and Circadian Rhythms: The Role of Melatonin and Adenosine in Zebrafish

Thesis by

Avni Gandhi

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For Veronica

&

My Parents

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Abstract

Sleep is a highly conserved behavioral state whose regulation is still unclear. In this thesis I first briefly introduce the known sleep circuitry and regulation in vertebrates, and why zebrafish is seen as a good model to study sleep-regulation. I describe the existing two-process model of sleep regulation, which posits that the two processes C (circadian) and S (homeostatic) control timing of sleep-wake behavior. I then study the role melatonin plays in the circadian regulation of sleep using zebrafish. First, we find that the absence of melatonin results in a reduction of sleep at night, establishing that endogenous melatonin is required for sleep at night. Second, melatonin mutants show a reduction in sleep in animals with no functional behavioral rhythms suggesting that melatonin does not require intact circadian rhythms for its effect on sleep. Third, melatonin mutants do not exhibit any changes in circadian rhythms, suggesting that the circadian clock does not require melatonin for its function. Fourth, we find that in the absence of melatonin, there is no rhythmic expression of sleep, suggesting that melatonin is the output molecule of process C. Finally, we describe a connection between adenosine signaling (output molecules of process S), and melatonin. Following this we proceed to study the role adenosine signaling plays in sleep-wake behavior. We find that first, adenosine receptor A1 and A2 are involved in sleep- wake behavior in zebrafish, based on agonist/antagonist behavioral results. Second, we find that several brain regions such as PACAP cells in the rostral midbrain, GABAergic cells in the forebrain and hindbrain, Dopamine and serotonin cells in the caudal hypothalamus and sox2 cells lining the hindbrain ventricle are activated in response to the A1 antagonist and VMAT positive cells are activated in response to the A2A agonist, suggesting these areas are involved in adenosine signaling in zebrafish. Third, we find that knocking out the zebrafish adenosine receptors has no effect on sleep architecture. Finally, we find that while the A1 agonist phenotype requires the zfAdora1a receptor, the antagonist and the A2A agonist behavioral phenotypes are not mediated by the zfAdora1a, zfAdora1b and zfAdoraA2Aa, zfAdora2Ab receptors respectively.

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Chapter 1

Introduction

Introduction

Melatonin and adenosine are two small molecules that have been reported to have an important effect on sleep wake behavior. This study examines how these two molecules individually contribute to the regulation of sleep-wake behavior and proposes a model by which one regulates the other to bring about the sleep/wake states. In order to study their role on sleep, a scientist's definition of sleep is first required. Sleep can be a defined as a rapidly reversible state of behavioral quiescence which must have the following features to be called sleep: (1) consolidated periods of immobility exhibiting circadian rhythmicity (2) a specific posture, specific to individual species (3) a decrease in sensory responsiveness/ or an increased arousal threshold (4) a homeostatic regulatory mechanism or a mechanism by which deprivation of the behavior results in an accompanying rebound once the deprivation stimulus has been removed (Campbell and Tobler, 1984; Hendricks et al., 2000).

Using these criteria, sleep has been observed in a variety of the members of the animal kingdom including humans, zebrafish and fruit flies (Campbell and Tobler, 1984; Hendricks et al., 2000). Sleep deprivation assays have shown that prolonged deprivation of sleep results in deterioration of performance in memory related tasks. The absence of sleep can results in deterioration in performance and eventually prolonged deprivation can result in death (Everson et al., 1989; Li et al., 2009; Seugnet et al., 2008; Shaw et al., 2002). These results have lead scientists to theorize that during sleep episodes maintenance processes must be occurring in the brain, which are crucial to brain performance. One theory suggests that the purpose of sleep is to replenish depleted brain energy stores such as glycogen (Benington and Craig Heller, 1995). This theory places

low the low energy molecule adenosine in a central role, as a readout of the brain energy levels, and a regulator of sleep wake circuits (Benington et al., 1995). Another theory suggests that the purpose of sleep may be to trim the majority of the connections formed in the brain over the course of the day in order to be able to absorb new information the next day, and to also strengthen specific important connections, which has been supported by evidence from *drosophila* (Tononi and Cirelli, 2003) . In either case, regulating the occurrence of sleep then can be considered to be very important for animals.

What is the neural circuitry that regulates sleep? Studies show that the hypothalamic circuitry plays a major role in the control of sleep-wake behavior. There are two neural systems in the brain that control sleep-wake behavior. The first is the ascending arousal system situated in the brainstem/hypothalamus region. It includes a group of nuclei such as the locus coeruleus (Noradrenergic), Dorsal Raphae nucleus (Serotonergic), laterodorsal tegmental nucleus (Cholinergic), Pedunctulopontine nucleus (cholinergic) and Tuberomamillary nucleus (Histaminergic). The ascending arousal system also includes the orexinergic and melanin concentrating hormone neurons in the lateral hypothalamus, the cholinergic neurons in the basal forebrain and the dopaminergic neurons in the ventral periaqueductal gray region. The second system is the sleep active neurons in the ventero-lateral pre optic area (VLPO). The projections from these neurons inhibit the wake active areas, according to the flip-flop model of sleep regulation (Saper et al., 2001, 2005). At any one time, either the ascending arousal system is active or the sleep system is active since they inhibit each others, which results in sleep state and the wake state being mutually exclusive. The molecules that bring about this flip are unclear

however Adenosine has been proposed to be a candidate. (Basheer et al., 2000; Radulovacki et al., 1984; Saper et al., 2005).

What then controls the timing of the two states? According to the two-process model proposed by (Borbély, 1982), sleep is controlled by two processes, process C and process S. Process C is the circadian process, whose levels vary under the control of the circadian clock. It is regulated by external factors such as light. Process S is the homeostatic process, which responds to internal cues of sleep need. Its levels rise with wakefulness and decrease with sleep. While the molecular workings of process C have been well characterized, its output molecule was unknown. In contrast, for process S, there have been several candidates for the output molecule, including Adenosine, AMP, NOS (Brown et al., 2012), however where in the brain they are produced and how their release is regulated is quite unclear. Melatonin however is a good candidate for the process C output molecule. Its levels are under the control of the circadian clock in humans, rodents as well as in zebrafish (Klein, 2007). Also exogenous melatonin induces sleep in vertebrates (Cajochen et al., 1997, 2003; Zhdanova et al., 1996).

We decided to use zebrafish to study the role played by melatonin and adenosine in sleep wake behavior. Why use zebrafish? Zebrafish is a comparatively new model system developed in the 1970s. Zebrafish can be housed compactly; moreover they produce many hundreds of embryos at a time. They are amenable to genetic manipulations and they are transparent, making them ideal for imaging. In addition to all these characteristics, they also exhibit the hallmarks of sleep behavior by the age of 4dpf (Prober et al., 2006; Zhdanova, 2006). Zebrafish are vertebrates, but they possess a simpler nervous system than mammals. They possess the components of the sleep wake

circuitry in higher vertebrates (Chiu and Prober, 2013), but they have lesser numbers of neurons, for example only 10-12 orexin neurons as compared to the over 100 neurons in rats (Liu et al., 2015; Prober et al., 2006). They also exhibit similar responses to agonists and antagonists as higher vertebrates (Rihel et al., 2010). The zebrafish blood brain barrier is formed during 3-10 days post fertilization. There is therefore, good brain penetrance of sleep-wake affecting drugs added directly to the fish-water, during this period (Fleming et al., 2013). The small size of zebrafish larvae allows for high-throughput behavioral assays to be performed, which allows for statistically solid behavioral experiments (Kokel and Peterson, 2008; Rihel et al., 2010). Moreover they have a fully functional circadian system at 5dpf (Hirayama et al., 2005; Kazimi and Cahill, 1999). Also zebrafish are amenable to the ZFN, TALEN and CRISPR technologies, making it possible to generate desired gene mutants (Chen et al., 2013; Hwang et al., 2013; Sander et al., 2011a, 2011b). All these advantages result in the zebrafish model system being well poised to answer many questions relating to sleep-wake regulation.

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Chapter 2

Melatonin is required for the circadian regulation of sleep

Abstract

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.

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Introduction

Sleep is an evolutionarily conserved behavioral state whose regulation is poorly understood (Sehgal and Mignot, 2011). A key model postulates regulation by a homeostatic process that responds to internal cues for sleep need and a circadian process that responds to external cues (Borbély, 1982). Several lines of evidence suggest that accumulation of extracellular adenosine in specific brain regions plays an important role in the homeostatic mechanism, although it is clear that other factors are also required (reviewed in Brown et al., 2012). However, while mechanisms that regulate the circadian clock are well characterized (Fisher et al., 2013), molecules that transmit circadian information to regulate sleep are largely unknown.

Melatonin is an attractive candidate for mediating the circadian process because the clock regulates its production (Klein, 2007) and it induces sleep in some contexts (Fisher et al., 2013). However, despite decades of study and widespread use, the role of melatonin in regulating sleep is controversial (Fisher et al., 2013). Exogenous melatonin is reported to have sleep-promoting effects in diurnal vertebrates including humans (Brzezinski et al., 2005; Zhdanova, 2005), nonhuman primates (Zhdanova et al., 2002), domesticated cats (Goldstein and Pavel, 1981), birds (Mintz et al., 1998), and zebrafish (Zhdanova et al., 2001). However, others failed to observe this effect (Buscemi et al., 2006; van den Heuvel et al., 2005), and its endogenous role in any animal is unknown. Exogenous melatonin can entrain (Lockley et al., 2000; Sack et al., 2000) and phase shift (Lewy et al., 1992) the circadian clock in some contexts, and melatonin has been called a regulator of circadian rhythms (Elbaz et al., 2013). Indeed, melatonin has been proposed to promote sleep indirectly by phase advancing the circadian clock (Arendt, 2003) or by

inhibiting the circadian drive for wakefulness (Scheer and Czeisler, 2005). However, these hypotheses are based on exogenous melatonin. Determining melatonin's role in sleep is complicated by its production at night in both diurnal and nocturnal animals, and because most laboratory mouse strains produce little or no melatonin (Goto et al., 1989). As a result, studies have relied on pharmacological approaches that have produced inconsistent results (Fisher et al., 2013). To determine whether endogenous melatonin regulates sleep in a diurnal vertebrate, we analyzed melatonin function in the zebrafish, whose circadian clock and sleep regulation are conserved with mammals (Elbaz et al., 2013).

Results

Melatonin promotes initiation and maintenance of night-time sleep

Melatonin is produced in the zebrafish pineal gland at night under control of the circadian clock (Kazimi and Cahill, 1999), as in mammals. To determine whether endogenous melatonin is required for sleep, we generated zebrafish with a predicted null mutation in *arylalkylamine N-acetyltransferase 2* (*aanat2*) (Figure 2.2A), which is required for melatonin synthesis in the pineal gland (Klein, 2007). We found that wild-type (WT) larvae in 14:10 hr light:dark (LD) conditions had high melatonin levels at night and low levels during the day, while *aanat2* homozygous mutants *(aanat2*$^{-/-}$) produced little to no melatonin (Figure 2.2B). To determine whether melatonin is required for sleep, we used a videotracking assay (Prober et al., 2006) to compare sleep/ wake behaviors of *aanat2*$^{-/-}$ larvae to their *aanat2*$^{+/-}$ and WT siblings. We found that all three genotypes exhibited similar day- time amounts of sleep and activity (Figures 2.1A–C and E). However, at night, *aanat2*$^{-/-}$ larvae slept almost half as much and were three times more active than controls (Figures 2.1A,B, D, F). Decreased nighttime sleep was due mainly to a decrease in sleep bout length and a corresponding increase in wake bout length, with little effect on bout number (Figure 2.3B,D,H). Sleep latency (time between lights-out and sleep) at night was also longer for aanat2$^{-/-}$ larvae (Figure 2.3J). These results demonstrate that endogenous melatonin promotes initiation and maintenance of nighttime sleep in a diurnal vertebrate.

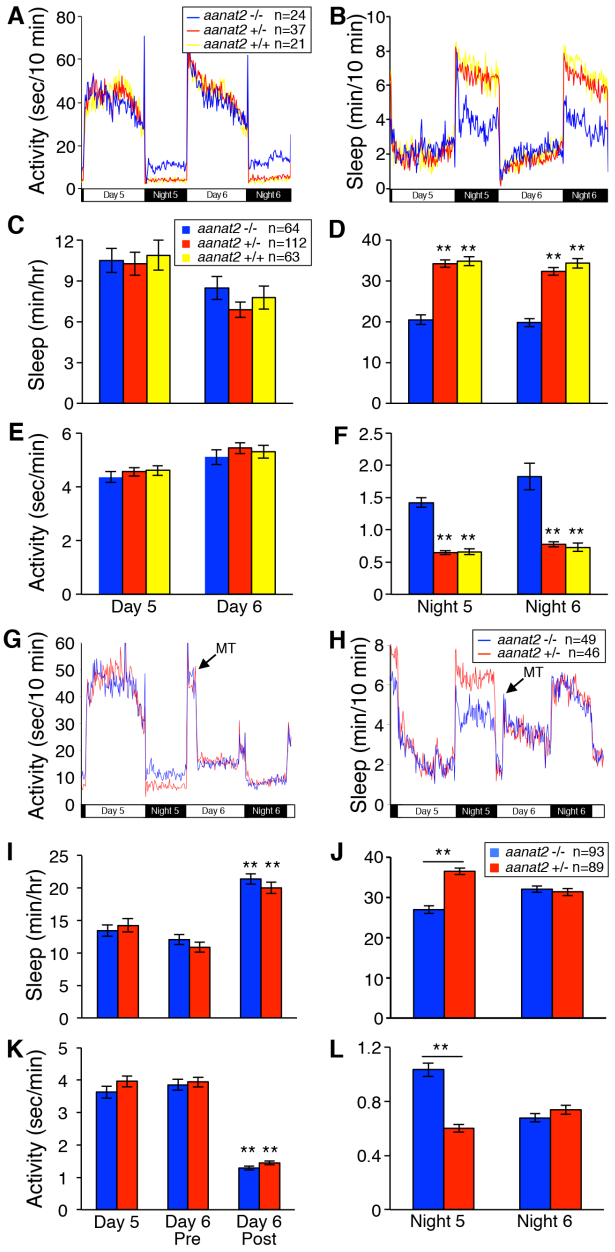

Figure 2.1 *aanat2-/-* larvae sleep less at night. (A-F) *aanat2-/-* larvae sleep less (B,D) and are more active (A,F) than sibling controls at night, but not during the day (A,B,C,E). (G-L) Arrow indicates addition of 10µM melatonin on day 6. *aanat2-/-* sleep and locomotor activity phenotypes of night 5 are absent on night 6 (G,H,J,L). During the day, exogenous melatonin decreases locomotor activity (G,K) and increases sleep (H,I) for both genotypes. Day 6 Pre and Post refer to periods before and after melatonin addition. Total sleep amount can vary for nights 5 and 6 of development, so comparisons between different genotypes should be made on the same night. Data are from one representative experiment (A-B,G-H) or combined from two (I-L) or three (C-F) experiments. Bar graphs represent mean ± SEM. n, number of larvae. **, $p<0.01$ compared to aanat2-/- (C-F), each genotype Day 6 Post compared to Day 6 Pre (I,K), or the indicated comparisons (J,L) by Dunnett's (C-F) or Tukey's test (I-L). See also Figure 2.3.

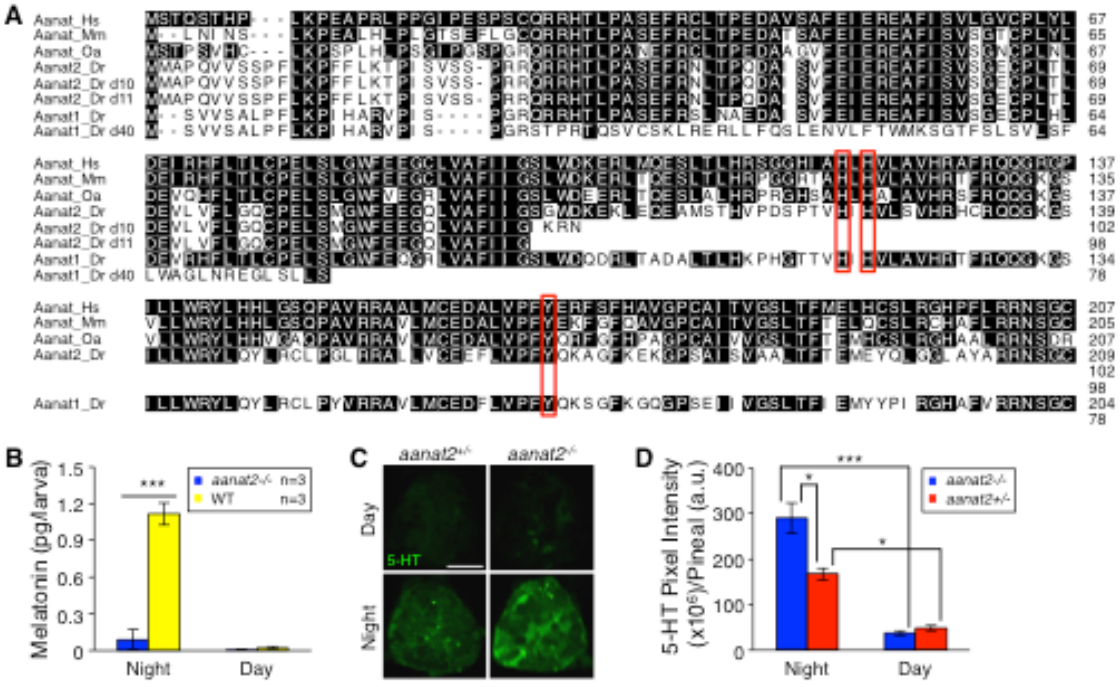

Figure 2.2 *aanat* mutant sequences and effects *aanat2* mutation on melatonin and serotonin levels (A) Amino acid sequence alignment of human (Hs), mouse (Mm) and sheep (Oa) Aanat, and zebrafish (Dr) Aanat1 and Aanat2, are shown. The sequences of two zebrafish Aanat2 mutant proteins (d10 and d11) and one zebrafish Aanat1 mutant protein (d40) that were generated in this study are also shown. Red boxes indicate conserved amino acids that interact with the acetyl-CoA substrate and are critical for enzyme activity in the sheep Aanat protein (Hickman et al.,1999; Scheibner et al., 2002). (B) *aanat2-/-* larvae contain little or no melatonin. Melatonin concentration was assayed by ELISA from WT and *aanat2-/-* larvae at the end of the night (6 am) or end of the day (6 pm). Little or no melatonin was detected in WT larvae at 6 pm or in *aanat2-/-* larvae at either time point. The assay was performed in triplicate, with 5 larvae per sample. Mean ± SEM is shown. ***, p<1x10-6 by Student's t-test. (C, D) Serotonin (5-HT) levels in the pineal gland are higher at night compared to the day in both *aanat2+/-* and *aanat2-/-* larvae, and are higher at night in *aanat2-/-* larvae compared to their *aanat2+/-* siblings. Single 0.75 µm thick confocal sections are shown. Number of animals analyzed: *aanat2+/-* day=11, *aanat2+/-* night=6, *aanat2-/-* day=7, *aanat2-/-* night=13. Mean ± SEM is shown. *, p<0.05. ***, p<1x10-6 By Tukey's test. Scale=20 µm.

Aanat2 loss-of function phenotype is mediated by melatonin and not serotonin

Serotonin is acetylated by AANAT to form acetylserotonin, which is methylated to form melatonin. Loss of aanat2 may thus elevate serotonin levels in the pineal gland. Consistent with mammalian results (Borjigin et al., 2012), we found that serotonin levels are higher in the pineal. Serotonin levels were even higher at night in *aanat2*$^{-/-}$ larvae compared to their *aanat2*$^{+/-}$ siblings, indicating that loss of *aanat2* results in higher pineal serotonin levels (Figure 2.2 C,D). To test whether the *aanat2*$^{-/-}$ behavioral phenotype is due to loss of melatonin or increased serotonin, we added exogenous melatonin, which completely rescued the aanat2$^{-/-}$ phenotype (Figure 2.1 G-L), indicating that it is due to loss of melatonin.

Aanat1 is not required for sleep

Zebrafish have a second aanat ortholog (*aanat1*) that is expressed in the retina (Appelbaum et al., 2006). To test whether aanat1 is partially redundant with aanat2 in regulating sleep, we generated zebrafish with a predicted null mutation in aanat1 (Figure 2.2A). The behavioral phenotype of *aanat1*$^{-/-}$*; aanat2*$^{-/-}$ larvae was indistinguishable from that of *aanat2*$^{-/-}$ larvae (data not shown), suggesting that aanat1 is not required for sleep.

Aanat2 mutants exhibit normal sensory responsiveness/arousal

Since *aanat2*$^{-/-}$ larvae sleep less at night, we hypothesized that their arousal threshold might be reduced. To test this hypothesis, we applied a mechanoacoustic stimulus (Woods et al., 2014) at night at 1-minute intervals at a range of intensities. Surprisingly, we found that *aanat2*$^{-/-}$larvae and their sibling controls exhibited similar

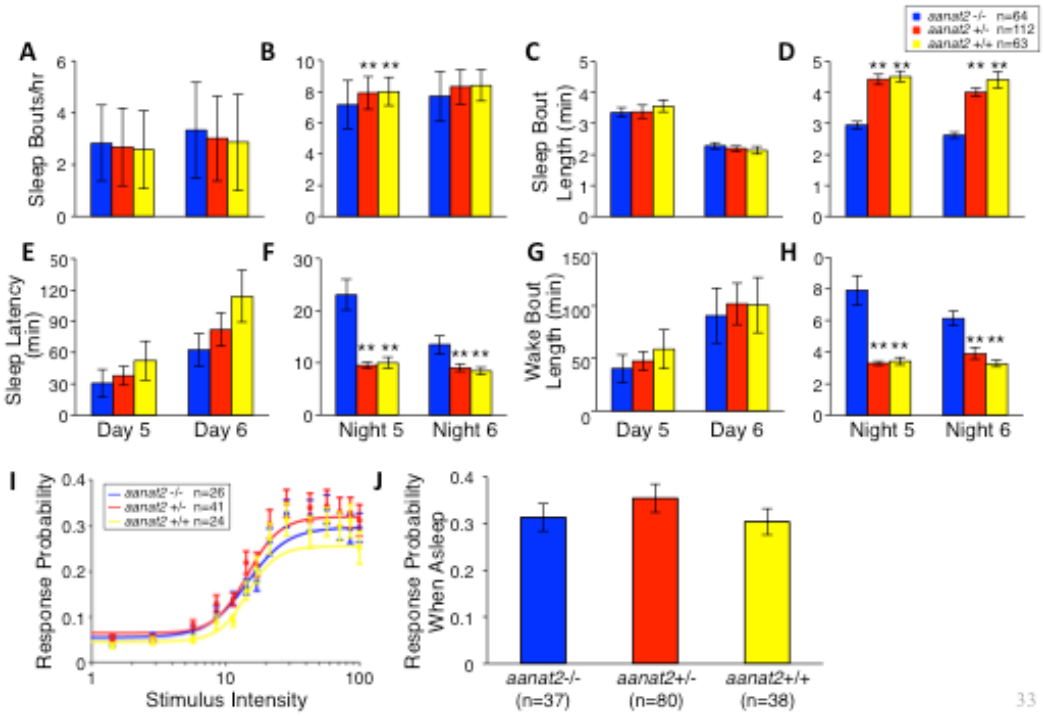

Figure 2.3 Effects *aanat2* mutation on behavior in LD and sensory responsiveness/arousal (Related to Figure 2.1). (A-H) *aanat2-/-* larvae are more active and sleep less at night in LD compared to their heterozygous mutant and WT (siblings. Graphs are based on the data shown in Figures 1A-1F. *aanat2-/-* larvae exhibit slightly fewer sleep bouts (B), shorter sleep bouts (D), longer sleep latency (F), and longer wake bouts (H) at night, but no phenotype during the day (A, C, E, G). Data is combined from three experiments. Median ± median absolute deviation (MAD) (A, B) and mean ± SEM (C-H) are shown. n, number of larvae analyzed. *, $p<0.05$; **, $p<0.01$ compared to *aanat2-/-* by Steel-Dwass (A, B) or Dunnett's test (C-H). (I) The response probability to a mechanoacoustic stimulus applied once per minute at night over a range of stimulus intensities is similar for all genotypes (log (half-maximal response probability) = 1.17±0.03, 1.15±0.03 and 1.16±0.04 for *aanat2-/-, aanat2+/-* and *aanat2+/+,* respectively; p=0.92 by extra sum-of-squares F test). Each data point represents the mean response probability ± SEM at a particular stimulus intensity. The x- axis shows 0% to 100% stimulus intensity plotted as arbitrary units on a logarithmic scale. (J) When taps were applied every 5 minutes, sleeping (inactive for 1 or more minutes) *aanat2-/-*, *aanat2+/-* and *aanat2+/+* larvae were equally likely to respond (response probability = 0.31±0.03, 0.35±0.03, and 0.30±0.03, respectively; p=0.43 by one-way ANOVA). Mean ± SEM is shown. n, number of larvae analyzed.

half-maximal response probabilities (log(probability) 1.17±0.03, 1.15±0.03 and 1.16±0.04 for *aanat2*$^{-/-}$, *aanat2*$^{+/-}$ and *aanat2*$^{+/+}$, respectively; p=0.92 by extra sum-of-squares F test) (Figure 2.3I), indicating that aanat2$^{-/-}$ larvae have a normal arousal threshold. We next asked whether sleeping *aanat2*$^{-/-}$ larvae are more likely to awaken in response to a stimulus, since light sleep is often a feature of insomnia. To test this hypothesis, we stimulated larvae every 5 minutes, allowing larvae to re-enter sleep after each stimulus. We used a tap strength at which 50% of larvae normally respond. There was no significant difference among the three genotypes (p=0.43 by one-way ANOVA) (Figure 2.3J). Thus, although aanat2$^{-/-}$ larvae sleep less at night, their arousal threshold and sleep depth are similar to controls, suggesting that their sleep defect is not caused by hyperarousal.

Pineal ablation results in increased activity and reduced sleep at night

As an alternative approach to deplete melatonin we ablated melatonin-producing cells. We generated transgenic zebrafish in which the *aanat2* promoter drives expression of cyan fluorescent protein fused to nitroreductase (CFP-NTR), a bacterial protein that converts the inert pro-drug metronidazole (MTZ) into a potent DNA cross-linking agent that causes cell- autonomous death (Curado et al., 2007). CFP-NTR was expressed exclusively in aanat2- expressing pineal gland cells (Figure 2.4A, B), which developed normally but underwent cell death upon addition of MTZ (Figure 2.4C-E). Some pineal gland cells died within a few hours of adding MTZ, but we observed the most robust ablation without non-specific drug toxicity with treatment from 60-80 and 108-128 hpf,

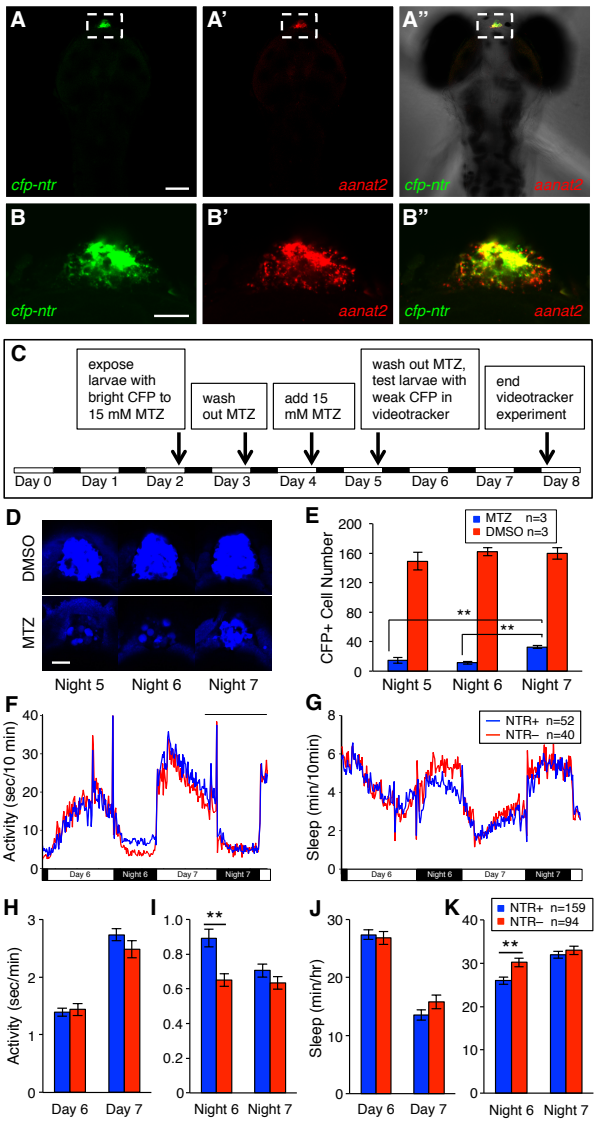

Figure 2.4. Depletion of aanat2-expressing pineal gland cells decreases sleep at night (Related to Figure 2.1). (A, B) Double fluorescent in situ hybridization using probes specific for cfp-ntr (green) and aanat2 (red) was performed on a Tg(*aanat2*:CFP-NTR) larva fixed at 120 hpf. Boxed region in (A) is shown at higher magnification in (B). Scale bar=100 μm (A) and 20 μm (B). cfp-ntr expression is only observed in aanat2-expressing pineal gland cells. (C) Ablation experiment outline. Larvae were treated with 15 mM MTZ from 60-80 and 108-128 hpf, and behavioral monitoring was initiated at 134 hpf. (D) aanat2:CFP-NTR expressing cells in the pineal gland were imaged during the fifth, sixth and seventh nights of development in larvae treated with DMSO vehicle control or MTZ. Scale bar=20 μm. (E) Quantification of *aanat2*:CFP-NTR cells. Mean ± SEM is shown. n, number of larvae quantified. (F-K) Tg(*aanat2*:CFP-NTR) larvae exhibit increased locomotor activity (F, I) and decreased sleep (G, K) during night 6 compared to non-transgenic siblings. The phenotype is absent on night 7, presumably due to the increased number of aanat2-expressing cells (D, E). Data are from one representative experiment (F,G) or 3 experiments combined (H-K). **, $p<0.01$ for the indicated comparisons by Tukey's test.

removing MTZ between treatments (Figure 2.4C-E). As observed for *aanat2*$^{-/-}$ larvae, *aanat2*-CFP-NTR larvae slept less and were more active at night (Figure 2.4F-K), although the effect was weaker, likely due to persistence of a small number of aanat2-expressing cells (Figure 2.4D,E). Consistent with this hypothesis, 48 hours after MTZ removal we observed a small number of new aanat2-expressing cells (Figure 2.4D, E) and larvae exhibited normal amounts of sleep and activity (Figure 3F-K), indicating that the aanat2-CFP- NTR phenotype is reversible and suggesting that a small number of pineal gland cells are sufficient for normal sleep.

Endogenous melatonin promotes sleep directly rather than via the circadian clock

Melatonin has been proposed to promote sleep indirectly, by phase-advancing the circadian clock (Arendt, 2003) or inhibiting the circadian drive for wakefulness (Scheer and Czeisler, 2005). To determine whether the sleep-promoting role of endogenous melatonin results from effects on the circadian clock, we raised and tested *aanat2*$^{-/-}$ larvae in the dark (DD), resulting in arrhythmic animals lacking overt behavioral or molecular circadian rhythms (Figures 2.5A-F and 2.6G-H) (Kaneko and Cahill, 2005). To determine whether the arrhythmia observed in DD is due to arrested or asynchronous cellular circadian clocks (Dekens and Whitmore, 2008) we performed fluorescent in situ hybridization for the clock gene per1b (Figure 2.6I-Q). If the lack of overt rhythms is caused by arrested clocks, per1b should be expressed similarly at all circadian time points. However, if it results from asynchronous clocks, expression should be more heterogeneous in larvae raised in DD than in those raised in LD. Furthermore, for asynchronous clocks, the average fluorescence intensity among many cells in DD should

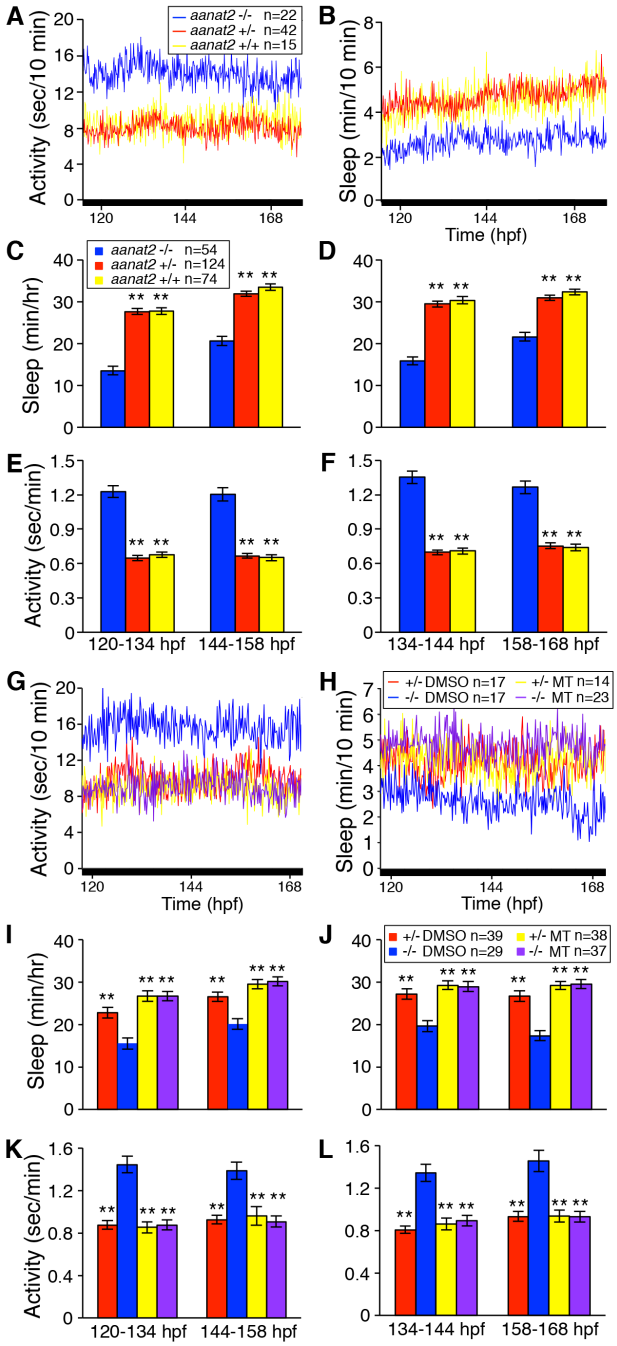

Figure 2.5. Reduced sleep in *aanat2-/-* zebrafish does not require an entrained circadian rhythm. (A,B) Larvae raised and tested in DD do not exhibit circadian locomotor activity or sleep rhythms. *aanat2-/-* larvae are more active (A,E,F) and sleep less (B-D) than sibling controls. (G-L) *aanat2-/-* larvae treated with vehicle control are more active (G,K,L) and sleep less (H-J) than *aanat*+/- siblings. *aanat2-/-* larvae treated with 10 μM melatonin exhibit locomotor activity and sleep indistinguishable from their melatonin-treated *aanat*+/- siblings (G-L). Data are from one representative experiment (A-B,G-H), or combined from two (I-L) or three (C-F) experiments. Bar graphs represent mean ± SEM for the 5th and 6th days and nights of development. **, $p<0.01$ compared to *aanat2-/-* (C-F) or *aanat2-/-* DMSO (I-L) by Dunnett's test. See also Figure 2.6

be lower than the peak level in LD. We found that per1b expression oscillates throughout the brain in LD (Figure 2.6I, K, M, O) with phasing consistent with data from reverse-transcription quantitative PCR (RT-qPCR) (Figure 2.6G). In contrast, per1b is expressed at a similar level at all circadian time points in larvae raised in DD (Figure 2.6J, L, N, P). Quantification of fluorescence intensity in the hypothalamus showed that average per1b levels in DD are similar to peak levels in LD (Figure 2.6Q). Quantification in the forebrain and midbrain produced similar results (data not shown). These results suggest that raising larvae in DD abolishes circadian rhythms at the cellular level. Under these conditions, *aanat2*$^{-/-}$ larvae exhibited continuously decreased sleep and increased activity (Figure 2.5A-F). The magnitude of the difference was similar to that observed at night in LD (Figure 2.1A-F), and was again due to a decrease in sleep bout length and corresponding increase in wake bout length (Figure 2.6A-F). This phenotype was also rescued by exogenous melatonin (Figure 2.5G-L). These results suggest that endogenous melatonin promotes sleep directly rather than via the circadian clock in zebrafish.

Melatonin is not required for behavioral or molecular circadian rhythms

Having established that melatonin is required for sleep at night, we next asked whether melatonin is required for normal circadian rhythms. Studies in humans, nonhuman primates and rodents have shown that exogenous melatonin can phase-shift (Lewy et al., 1992) and entrain (Lockley et al., 2000; Sack et al., 2000) the circadian clock. However, loss of the pineal gland, and thus melatonin production, produces inconsistent results (Arendt, 2003). While pinealectomy in some species of fish, birds and reptiles affects circadian rhythms (Underwood, 2001), it is unclear whether this is due to

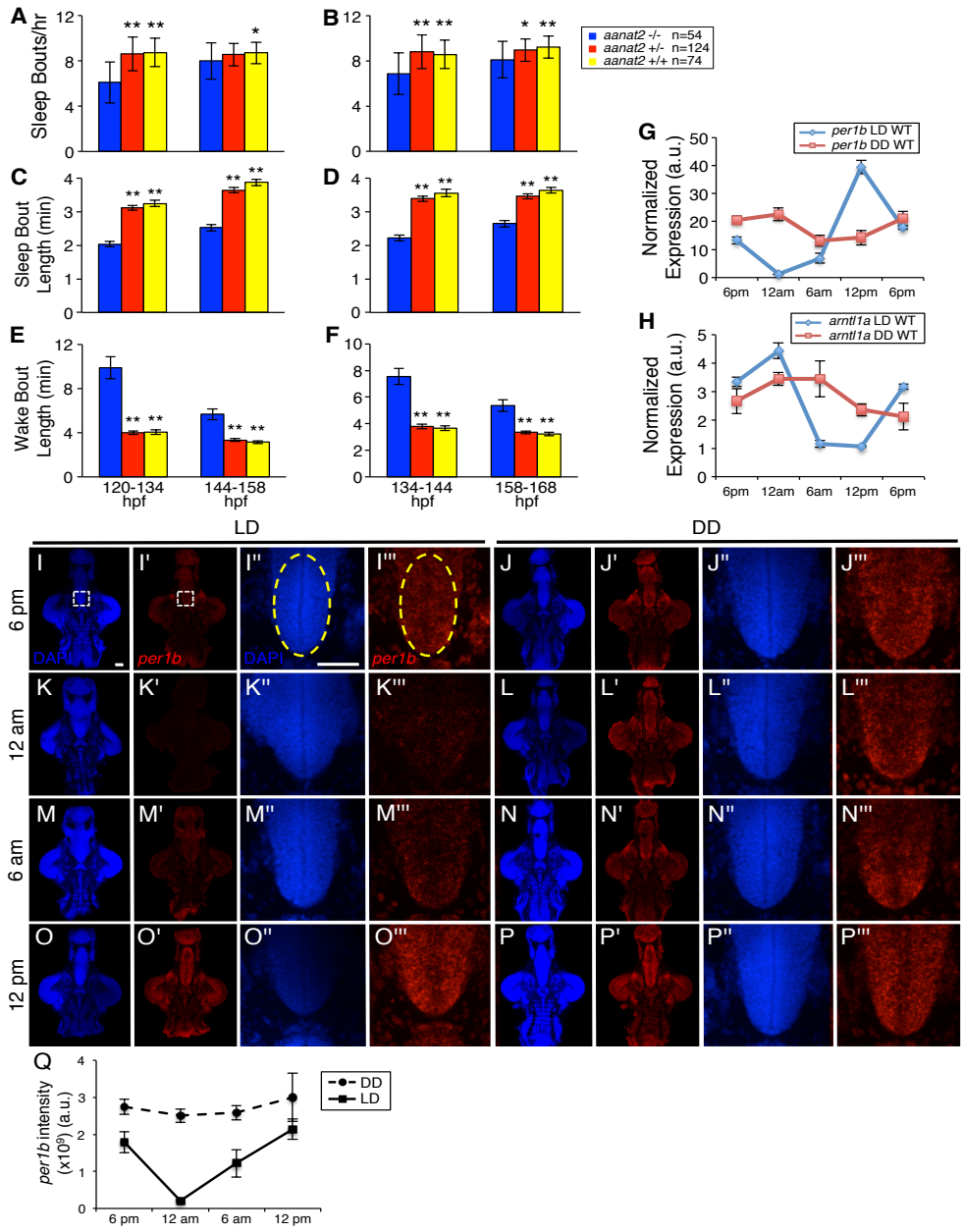

Figure 2.6. Reduced sleep in *aanat2-/-* zebrafish does not require an entrained circadian rhythm (Related to Figure 2.5). (A-F) Graphs are based on data shown in Figures 2A-2F. When raised and tested in DD, *aanat2-/-* larvae exhibit fewer sleep bouts (A, B), shorter sleep bouts (C, D) and longer wake bouts (E, F) during periods corresponding to the 5th and 6th days and nights of development. Data are combined from three experiments. Median ± MAD (A, B) and Mean ± SEM (C-F) are shown. n, number of larvae analyzed. *, $p<0.05$; **, $p<0.01$ compared to *aanat2- /-* by Steel-Dwass (A, B) or Dunnett's test (C-F). (G, H) WT larvae raised in DD (red) exhibit dramatically reduced or absent rhythmic expression of the circadian clock genes *per1b* (G) and *arntl1a* (H) compared to siblings raised in LD (blue). Samples were collected at 6-hour intervals from 6 pm on day 5 until 6 pm on day 6. The expression of both mRNAs display robust oscillations in LD but not in DD (peak:trough ratio is 33 in LD versus 2 in DD for *per1b*,

and 4.2 in LD versus 1.6 in DD for *arntl1a*). None of the DD data points for *per1b* or *arntl1a* is significantly different from any other ($p>0.12$ and $p>0.25$ for *per1b* and *arntl1a*, respectively, by Tukey's test). The LD data points are significantly different from each other ($p<1x10-6$ for *per1b* and *arntl1a* by Tukey's test). 24 larvae were pooled for each sample and the mean ± SEM of triplicate samples at each time point is shown. (I-Q). Larvae raised in LD (lights on at 9 am and off at 11 pm) or DD were fixed at the indicated time points beginning at 6 pm at 6 dpf. Dissected brains were processed for fluorescent *in situ* hybridization using a probe specific for *per1b* and were counterstained with DAPI to label nuclei. In LD, *per1b* expression oscillates with a peak at 12 pm and trough at 12 am (I, K, M, O). In DD, *per1b* expression levels are similar at all time points (J, L, N, P). White boxes in I and I' indicate region of the hypothalamus that is shown at higher magnification in (I"-P") and (I"'-P"'). Yellow ovals in I" and I"' indicate region used to quantify *per1b* fluorescence in all samples. Representative images are shown. Scale bar=50 μm. (Q). Quantification of total *per1b* fluorescence intensity in a 23 μm thick confocal image stack. *per1b* expression oscillates in LD (peak:trough ratio=10.7, $p<0.01$ by Tukey's test) but not in DD (peak:trough ratio=1.2, $p=0.77$ by Tukey's test). Mean ± SEM is shown. Three brains were quantified for each lighting condition at each time point.

loss of melatonin. We tested this using two approaches. We first asked whether melatonin is required for behavioral circadian rhythms using the videotracker assay. To monitor circadian rhythms in "free-running" conditions, we entrained larvae in LD and then shifted them to DD. In these conditions, WT larvae maintain molecular and behavioral rhythms (Figure 2.9A, B, K, L) (Kaneko and Cahill, 2005). While *aanat2*$^{-/-}$ larvae had smaller circadian amplitude due to increased locomotor activity during subjective night (Figures 6A,B and 8A, H), the period length and phase were similar to controls (Figure 2.7C, D). Second, we used transgenic fish in which the period 3 promoter regulates expression of luciferase (*per3*:luc), an in vivo reporter of molecular rhythms in intact larvae (Kaneko and Cahill, 2005). We found that *per3*:luc amplitude, period length and phase were not significantly different for any genotype (Figure 2.7E-H). To confirm this result, we isolated RNA from WT and *aanat2*$^{-/-}$ larvae and performed RT-qPCR for the circadian genes per1b and arntl1a. Circadian oscillation of both genes was indistinguishable between WT and *aanat2*$^{-/-}$ (Figure 2.9K,L), indicating that *aanat2*$^{-/-}$ larvae have normal molecular rhythms. Together the observation that *aanat2*$^{-/-}$ larvae exhibit normal locomotor activity period length and phase in LD (Figure 2.1A), this indicates that endogenous melatonin is not required to initiate or maintain circadian rhythms. We conclude that melatonin functions downstream of the circadian clock to promote sleep in zebrafish.

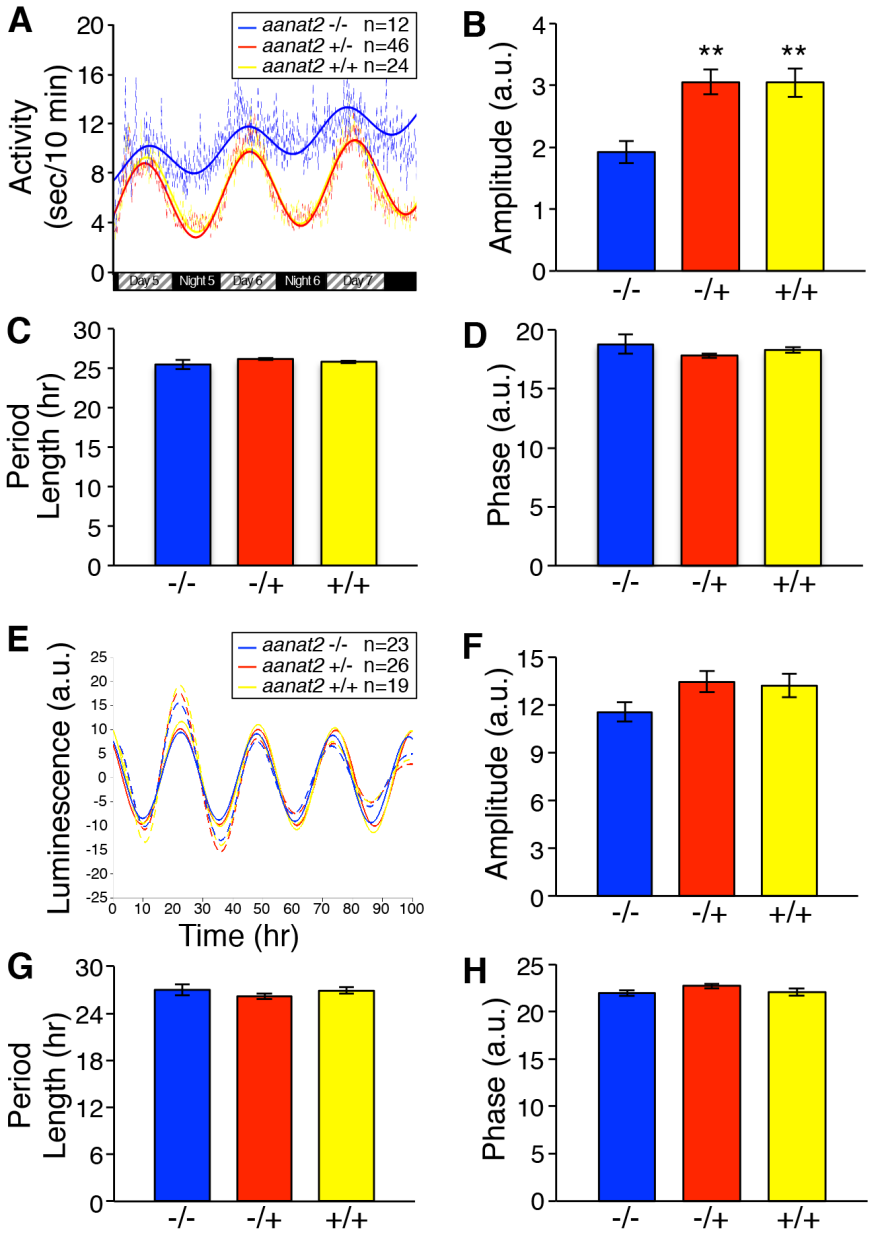

Figure 2.7. aanat2 is not required for behavioral or molecular circadian rhythms. (A) Larvae raised in LD and monitored in DD maintain locomotor activity circadian rhythms. Locomotor activity data (dashed lines) was fit to damped cosine curves (solid lines) to quantify circadian amplitude (B), period length (C) and phase (D). *aanat2-/-* larvae have reduced amplitude due to increased locomotor activity during subjective night, but period length and phase are indistinguishable from sibling controls. Black and hatched boxes indicate subjective night and day, respectively. (E-H) per3-luc larvae were raised in LD and luminescence recorded during 100 hours in DD. (E) Dashed and solid lines indicate luminescence data and damped cosine curve fits, respectively. (F-H) Circadian rhythm amplitude, period length and phase are not significantly different for any genotype. Bar graphs represent mean ± SEM. a.u., arbitrary units. **, $p<0.01$ compared to *aanat2-/-* by Dunnett's test. See also Figure 2.8.

Melatonin is required for the circadian regulation of sleep

Our results suggest that melatonin may mediate process C, which determines when sleep occurs during the circadian cycle (Borbély, 1982). To address this possibility, we entrained larvae in LD and transferred them to DD to remove masking effects of light on behavior. Under these conditions, *aanat2*$^{-/-}$ larvae, but not sibling controls, exhibited similar amounts of sleep during subjective day and night (Figure 2.8A-C), despite the maintenance of normal molecular rhythms (Figures 2.7E-F, 2.9K, L). This indicates that melatonin is required for circadian regulation of sleep. Combined with the observation that exogenous melatonin is sufficient to induce daytime sleep (Figure 2.1H,I) (Zhdanova et al., 2001), this suggests that melatonin mediates process C in zebrafish larvae.

Adenosine may act downstream of Melatonin to promote sleep at night

Exogenous melatonin can induce adenosine production in the mammalian forebrain (Zamorskii and Pishak, 2003). To test whether adenosine mediates sleep promotion by melatonin, we treated *aanat2*$^{-/-}$ larvae and their *aanat2*$^{+/-}$ siblings with the adenosine receptor agonist 5'-N- ethylcarboxamido-adenosine (NECA). NECA treatment decreased locomotor activity in *aanat2*$^{+/-}$ larvae during the day (Figures 2.8D and 2.10A) but had no effect on activity or sleep at night (Figure 2.8D-2.8G; arrows indicate transient artifacts caused by pipetting compounds into the plate), presumably because adenosine receptors are maximally activated at night. In contrast, NECA increased sleep and decreased activity at night for *aanat2*$^{-/-}$ larvae similarly to their *aanat2*$^{+/-}$ siblings, (Figure 2.8F,G), thus rescuing the mutant phenotype. As an alternative approach to modulate adenosine signaling, we treated larvae with ABT702, a small molecule inhibitor of

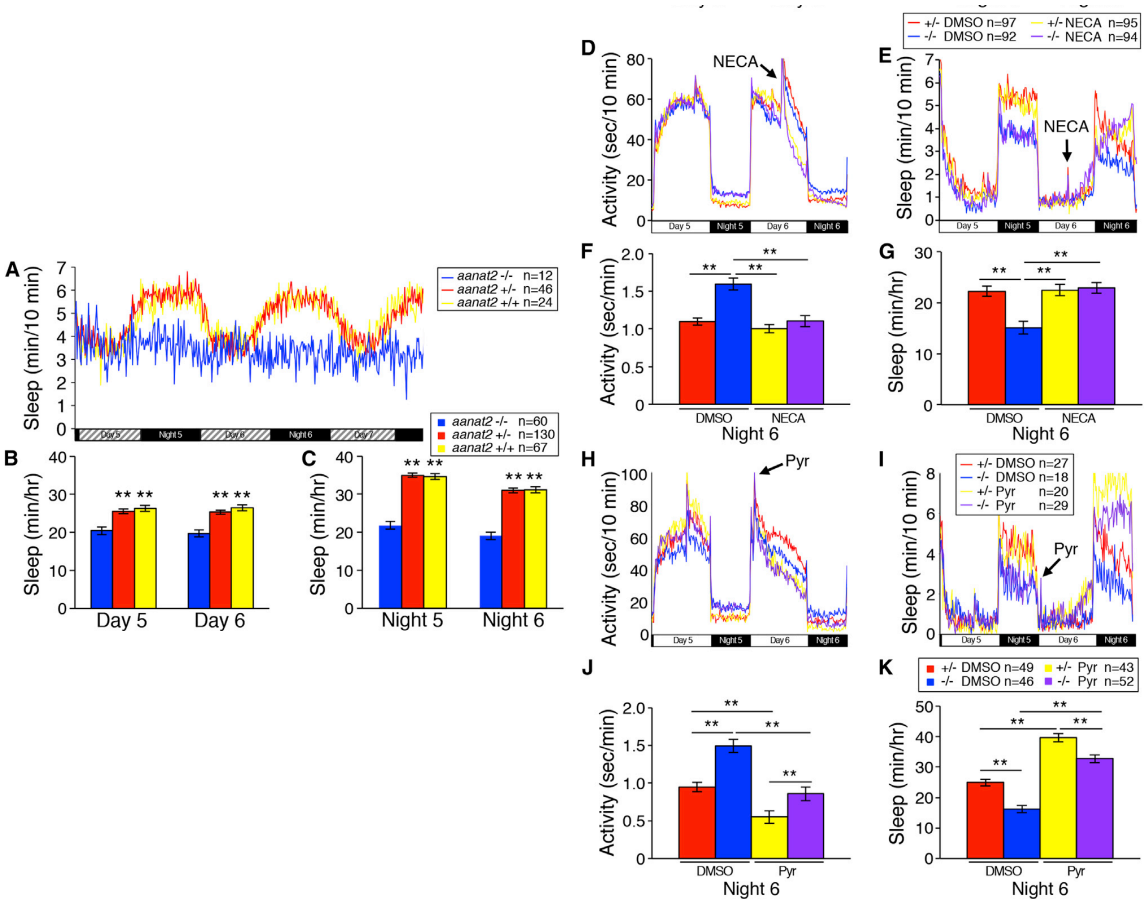

Figure 2.8. Melatonin is required for circadian regulation of sleep and may promote sleep via adenosine signaling. (A-C) Larvae were raised in LD and tested in DD. aanat2-/- larvae, but not sibling controls, lack circadian sleep oscillations. Black and hatched boxes represent subjective night and day, respectively. There is no significant difference in amount of sleep for aanat2-/-larvae during each subjective day (B) and night (C) ($p>0.22$ by Tukey's test). (D-G) Larvae were treated with vehicle or 50 µM NECA on day 6. On night 6, vehicle-treated aanat2-/- larvae are more active (D,F) and sleep less (E,G) than vehicle-treated aanat2+/- larvae. In contrast, NECA- treated *aanat2-/-* larvae exhibit the same amount of activity (D,F) and sleep (E,G) as NECA- treated *aanat2+/-* larvae. (H-K) Larvae were treated with vehicle control or 10 µM pyrilamine on day 6. Pyrilamine decreases locomotor activity (H,J) and increases sleep (I,K) for *aanat2-/-* and *aanat2+/-* larvae to a similar extent. Bar graphs represent mean ± SEM. **, $p<0.01$ compared to *aanat2-/-* (B,C), *aanat2-/-* DMSO (F,G), or for the indicated comparisons (J,K) by Dunnett's (B,C,F,G) or Tukey's test (J,K). Arrows indicate transient artifacts due to pipetting of compounds. See also Figure 8.

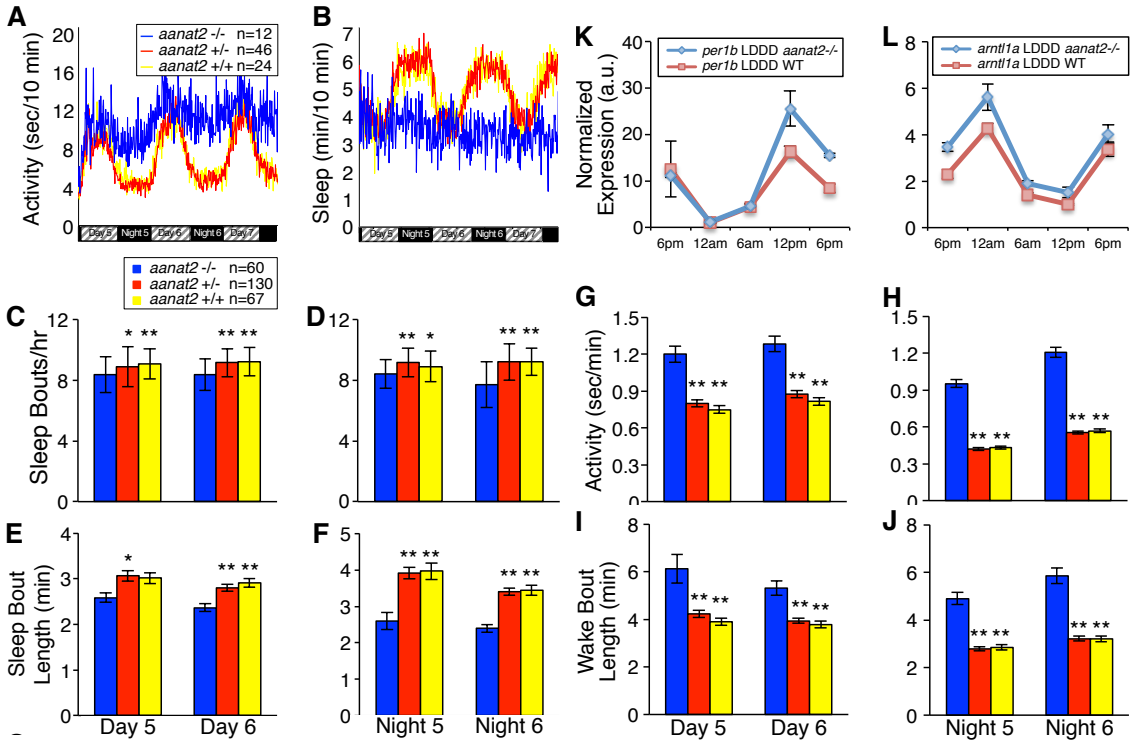

Figure 2.9. Melatonin is required for the circadian regulation of sleep (Related to Figure 2.8) (A-J) Graphs are based on data shown in Figures 7A-7C. (A, B) WT and heterozygous mutants raised in LD and tested in DD maintain locomotor activity and sleep circadian rhythms, but *aanat2-/-* larvae lack sleep circadian oscillations. Black and hatched boxes indicate subjective night and day, respectively. *aanat2-/-* larvae exhibit slightly fewer sleep bouts (D), shorter sleep bouts (F), more locomotor activity (H) and longer wake bouts (J) during subjective night. Similar but generally weaker phenotypes are observed during subjective day (C, E, G, I). Data are from one representative experiment (A, B) or combined from three (C-J) experiments. Bar graphs represent median ± MAD (C, D) and mean ± SEM (E-J). *, $p<0.05$; **, $p<0.01$ compared to *aanat2-/-* by Steel-Dwass (C, D) or Dunnett's test (E-J). (K, L) *aanat2-/-* and WT larvae raised in LD maintain circadian rhythms in DD (LDDD). WT (red) and *aanat2-/-* (blue) larvae were raised in LD and shifted to DD at 11 pm on the fourth night of development. Samples were collected at 6-hour intervals from 6 pm on day 5 until 6 pm on day 6. The expression of *per1b* (K) and *arntl1a* (L) oscillates for both WT and *aanat2-/-* larvae (peak:trough ratio is 16 for WT and 22 for *aanat2-/-* for *per1b*, and 4.3 for WT and 3.7 for *aanat2-/-* for *arntl1a*). 24 larvae were pooled for each sample and the mean ± SEM of triplicate samples at each time point is shown.

adenosine kinase that increases extracellular adenosine levels, and thus adenosine signaling (Brown et al., 2012). Similar to NECA, ABT702 (Figure 2.10B) had no effect on locomotor activity or sleep of *aanat2*$^{+/-}$ larvae at night, but rescued the activity and sleep phenotypes of *aanat2*$^{-/-}$ larvae (Figure 2.10D-F). These results suggest that melatonin may promote sleep, at least in part, by inducing adenosine signaling. To test whether these drugs failed to increase sleep at night in *aanat2*$^{+/-}$ larvae because they were already sleeping maximally, we promoted sleep via a different mechanism. Histamine is an arousing neuromodulator via mechanisms that are not well understood (Panula and Nuutinen, 2013). Inhibition of the histamine 1 receptor (H1R) is sedating in mammals (Panula and Nuutinen, 2013) and zebrafish (Renier et al., 2007). In contrast to NECA and ABT702, the H1R antagonist pyrilamine increased sleep and decreased activity in both *aanat2*$^{-/-}$ and *aanat2*$^{+/-}$ larvae to a similar extent (Figures 2.8H-K and 2.10C). This indicates that the failure of NECA to increase nighttime sleep in *aanat2*$^{+/-}$ larvae was not because the larvae were already sleeping maximally, and suggests that histamine acts parallel to, rather than downstream of, melatonin in regulating sleep. Together, our results are consistent with a model in which adenosine acts downstream of melatonin to promote sleep at night, thus potentially linking homeostatic and circadian regulation of sleep (Figure 2.11).

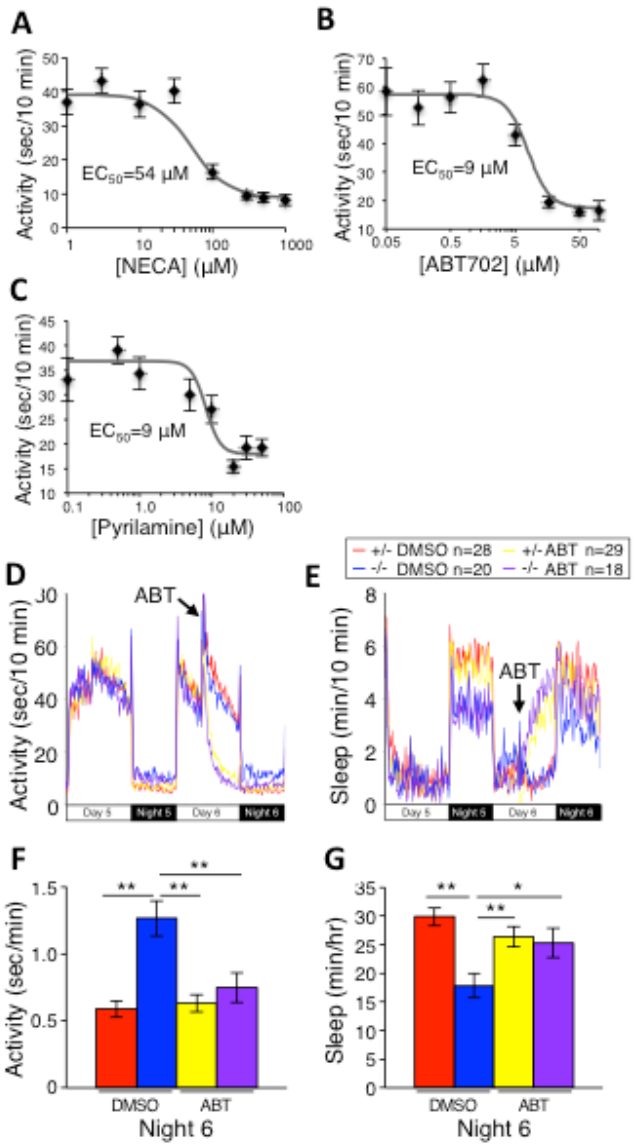

Figure 2.10. Drug dosage curves and melatonin may act through adenosine for its effects on sleep (Related to Figure 2.8) (A-C) Dose response curves for NECA (A), ABT702 (B) and pyrilamine (C). Each data point represents the average locomotor activity of 8 larvae during the day following addition of each drug. Drugs were used at or above the EC50 concentration for behavioral experiments. (D-G) Larvae were treated with either vehicle or 30 µM ABT702 starting on day 6. On night 6, vehicle-treated *aanat2-/-* larvae are more active (D, F) and sleep less (E, G) than vehicle-treated *aanat2+/-* larvae. In contrast, ABT702-treated *aanat2-/-* larvae exhibit the same amount of activity (D, F) and sleep (E, G) as ABT702-treated *aanat2+/-* larvae. Bar graphs represent mean ± SEM. *, $p<0.05$; **, $p<0.01$ compared to *aanat2-/-* DMSO by Dunnett's test. Note that there are no significant differences in locomotor activity or sleep on night 6 between ABT702-treated *aanat2+/-* and *aanat2-/-* larvae, but there are significant differences between DMSO- and ABT702-treated *aanat2-/-* larvae. Also note that the transient apparent increase in locomotor activity observed upon adding the drug or vehicle is an artifact due to compounds being pipetted into the plate.

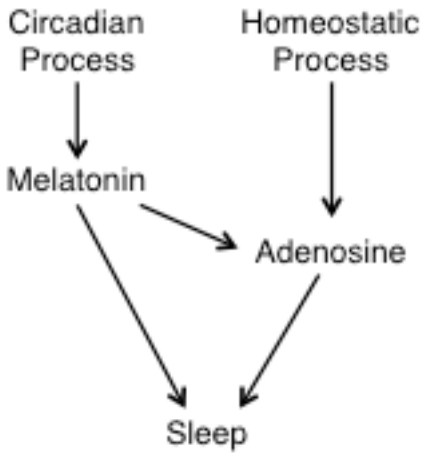

Figure 2.11. Melatonin is required for the circadian regulation of sleep and may act via adenosine (model) (Related to Figure 2.8). Model for circadian regulation of sleep by melatonin. The circadian clock regulates expression of *aanat2*, and thus melatonin production, in the pineal gland, with high levels at night and low levels during the day (Fisher et al., 2013). The high level of melatonin at night promotes the initiation and maintenance of sleep. Extracellular adenosine accumulates in specific brain regions in proportion to time spent awake, and is thought to play a role in the homeostatic regulation of sleep (Brown et al., 2012). Melatonin may promote sleep, at least in part, by promoting adenosine signaling, thus linking homeostatic and circadian regulation of sleep. Melatonin likely also promotes sleep via additional mechanisms because extracellular adenosine levels decline during sleep (Brown et al., 2012) whereas the *aanat2* mutant phenotype persists throughout the night. Melatonin-induced adenosine signaling may predominantly occur at the beginning of the night, when melatonin levels are still rising, to promote a rapid and robust wake to sleep transition. We found no evidence that melatonin feeds back to the circadian clock to regulate circadian rhythms.

Discussion

Melatonin is widely used as a sleep aid and has been cited as a regulator of circadian rhythms (Elbaz et al., 2013). However, despite decades of study, the role of melatonin in regulating sleep and circadian rhythms is controversial (Fisher et al., 2013) and the function of endogenous melatonin is unknown. Here we describe the first diurnal vertebrate genetic loss of function model for melatonin. We show that *aanat2*$^{-/-}$ zebrafish larvae take twice as long to fall asleep and sleep only half as much 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.

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. Indeed, while 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.

A prominent model proposes 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 (Figures 2.1G- L) (Zhdanova et al., 2001), and 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.

Because melatonin is produced at night in diurnal and nocturnal animals, and administration of physiological levels of melatonin does not promote sleep in nocturnal animals (Fisher et al., 2013), nocturnal and diurnal animals likely use different mechanisms for circadian regulation of sleep (Zhdanova, 2005). This idea is supported by the observation that most nocturnal mouse strains used for genetic studies produce little or no melatonin, (Goto et al., 1989) yet have circadian control of sleep. Mutation of *aanat* in melatonin-proficient nocturnal and diurnal mammals will clarify melatonin's role in regulating mammalian sleep.

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).

Finally, we note that reduced melatonin levels are associated with aging, mood disorders and autism (Hardeland, 2012). An improved understanding of how endogenous melatonin interacts with the sleep system and other aspects of physiology from the aanat2 mutant may lead to novel therapies for these disorders.

Materials and methods

Zebrafish Genetics

aanat2 mutant zebrafish were generated using the TAL effector nuclease (TALEN) method as described (Reyon et al., 2012) using plasmids obtained from Addgene. The TALEN target sites were 5'-TGGTGGCCTTCATCATT-3' and 5'-TGTTCTAGTTTCTCTT-3'. Two mutants were isolated and tested. Mutant d10 contains a 10 bp deletion (nucleotides 295-304 of the open reading frame: 5'-TCTGGCTGGG-3'). Mutant d11 contains an 11 bp deletion (nucleotides 296- 306 of the open reading frame: 5'-CTGGCTGGGAT-3'). The mutations result in a change in reading frame after amino acid 98 and a premature stop codon after amino acid 102 (d10) or 98 (d11), compared to 210 amino acids for the WT protein. The mutants exhibited identical phenotypes and mutant d10 was used for all reported experiments. aanat2 mutants were genotyped using the primers 5'-CAGTGTCCTGAGCTGTCCAT-3' and 5'-CGAAAGGCACATCACCATC-3'. Mutant and WT PCR products (162 bp and 172 bp, respectively) were distinguished by running the PCR reaction on a 4% agarose gel. aanat1 mutants were generated using the Cas9/CRISPR method (Hwang et al., 2013) using plasmids obtained from Addgene and the sgRNA 5'-GGAGCGTGTGTCGGCGCTGG-3'. The aanat1 mutant contains a 40 bp deletion (nucleotides 67-106 of the open reading frame: 5'-CGCCAGCGCCGACACACGCTCCCAGCAAGCGAGTTTCGCT-3'). This deletion results in a change in reading frame after amino acid 23 and a premature stop codon after amino acid 78, compared to 204 amino acids for the WT protein. aanat1 mutants were genotyped using the primers 5'-GTAGTGAGCGCACTGCCTTT-3' and 5'-

CACACATATGACCACACAAACCT- 3'. Mutant and WT PCR products (168 bp and 208 bp, respectively) were distinguished by running the PCR reaction on a 3% agarose gel. For both aanat1 and aanat2 mutants, the frame shifts and premature stop codons are 5' to domains critical for enzyme activity in the sheep aanat ortholog (Hickman et al., 1999; Scheibner et al., 2002), indicating that the mutant proteins are likely to be non-functional. The Tg(aanat2:CFP-NTR) transgenic line was generated by subcloning the 5' and 3' aanat2 enhancer elements (Gothilf et al., 2002) 5' and 3' to a CFP-NTR transgene (Curado et al., 2007) in a vector containing Tol2 transposase arms. The plasmid was coinjected with Tol2 transposase mRNA and transgenic lines were identified based on CFP fluorescence.

Behavioral Analysis

Larval zebrafish were raised on a 14:10 hour light:dark cycle at 28.5OC 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 of E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4) (Prober et al., 2006). In most experiments, plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. The sealing process introduces air bubbles in some wells, which are discarded from analysis. 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 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 28.5OC. The parameters used for detection were: detection threshold, 15; burst, 25; freeze, 3; bin size, 60 seconds. Larvae were exposed to 10 μM melatonin (3550, Tocris Bioscience) or 10 μM pyrilamine (P5514, Sigma Aldrich) by adding 100 μL of a 65 μM stock solution (in 0.02% and 0.065% DMSO for melatonin and pyrilamine, respectively) to E3 embryo medium during the experiment. Larvae were exposed to 50 μM NECA (E2387, Sigma Aldrich) by adding 100 μL of a 325 μM stock solution containing 1% DMSO to E3 embryo medium. Larvae were exposed to 30 μM ABT 702 dihydrochloride (2372, Tocris) by adding 100 μL of a 195 μM stock solution containing 0.65% DMSO to E3 embryo medium. The same concentration of DMSO was used as vehicle control for each drug experiment. For dose-response experiments, curve fitting was performed using a 4 parameter logistic nonlinear curve model and EC50 values were calculated using JMP 10.0 (SAS Institute Inc.).

Arousal Threshold Assay

The videotracking system was modified with an Arduino (http://www.arduino.cc/) based automated driver to control a solenoid (28P-I-12, Guardian Electric) delivering a tap to a 96-well plate containing larvae. Taps were applied from 12:30 am to 7:30 am the 5th night of development with an inter-trial-interval of 1 minute to measure overall arousal threshold or 5 minutes to assay arousal during sleep. Previous studies showed that a 30 second interval between repetitive stimuli is sufficient to prevent behavioral habituation

(Woods et al., 2014). In experiments where stimulus intensities were varied, 14 different intensities were applied in a random manner, with 30 trials at each intensity. For experiments where the stimulus was applied every 5 minutes, 60 trials were performed. The response of larvae to the stimuli was monitored using the videotracking software and subsequently analyzed in Matlab (version R2013a, TheMathworks, Inc) and Excel (Microsoft). Statistical analysis was performed using the Variable Slope log(dose) response curve fitting module of Prism (Graphpad).

period3-luciferase Assay

This assay was performed as described (Kaneko and Cahill, 2005), with some modifications. Larval zebrafish harboring a per3-luc reporter (Kaneko and Cahill, 2005) were raised on a 14:10 hour light:dark cycle at 22°C with lights on at 9 am and off at 11 pm. At 6 days post-fertilization (dpf), individual larvae were placed in each well of 96-well plates (T-2996-075, Greiner) containing 50 μL Holtfreter's solution (59 mM NaCl, 0.67 mM KCl, 0.76 mM CaCl2, and 2.4 mM NaHCO3, pH 7.0), 0.5 mM D-luciferin (L8220, Biosynth Chemistry and Biology) and 0.013% Amquel Instant Water Detoxifier (Kordon), and sealed with an optical adhesive film (4311971, Applied Biosystems). Bioluminescence in each well was recorded for 3 seconds every hour for 100 hours in constant darkness using a plate reader (M1000 Pro, Tecan).

Data analysis

Videotracker sleep/wake data was processed using custom PERL and Matlab (version R2013a, The Mathworks, Inc) scripts. Any one minute period with no movement was

defined as one minute of sleep (Prober et al., 2006). A sleep bout was defined as a continuous string of sleep minutes. Sleep latency was defined as the length of time from lights on or off to the start of the first sleep bout. Average activity was defined as the average amount of detected activity in seconds/minute, including all rest bouts. These parameters were calculated for each experimental day and night. Luminescence and behavioral data was processed for circadian analysis using custom Matlab scripts. Raw data were fit to a damped cosine curve as described (Hirota et al., 2008). Statistical tests were performed using JMP 10.0 (SAS Institute Inc.).

Pineal Ablation

Cell ablation using nitroreductase (NTR) was performed as described (Curado et al., 2007) with some modifications. aanat2-CFP-NTR lines containing multiple copies of the transgene and exhibiting high levels of CFP fluorescence in the pineal gland, with no non-pineal expression, were selected and incrossed. Embryos were raised in E3 medium until 60 hours post-fertilization (hpf), at which point they were separated into pools of larvae that exhibited strong or no CFP expression. Each pool was treated with 15 mM metronidazole (MTZ, 46461, Sigma Aldrich) in 0.2% DMSO/E3 medium for 20 hours (60-80 hpf). Larvae were then thoroughly rinsed with fresh E3 medium and maintained in E3 medium for 24 hours, followed by a second treatment with 15 mM MTZ for 20 hours (108-128 hpf). Larvae were then transferred to fresh E3 medium and screened for efficacy of pineal ablation using a fluorescence stereomicroscope (m205c, Leica Microsystems Inc.). Larvae with the weakest CFP fluorescence, as well as CFP negative siblings that were similarly treated and screened, were selected for behavioral monitoring

that was initiated at 134 hpf. To quantify the extent of pineal ablation, aanat2-CFP-NTR animals treated with MTZ or DMSO vehicle control as described above were anesthetized (0.016% w/v tricaine methane sulfonate, A5040, Sigma Aldrich) and mounted in 0.8% low melting agarose (16520-050, Invitrogen), and imaged on a confocal microscope (Zeiss 780 LSM, 20x dipping objective with 3x digital zoom). Imaging was performed between 11 pm and 7 am. The number of CFP-expressing cells was counted using the contrast between the nucleus and cytoplasm to distinguish individual cells for 3 animals at each time point. Double-fluorescent in situ hybridizations were performed using digoxygenin (DIG)- and 2,4-dinitrophenol (DNP)-labeled riboprobes and the TSA Plus DNP System (NEL741001, PerkinElmer). Samples were mounted in 50% glycerol/PBS and imaged using a confocal microscope (Zeiss 780 LSM).

Melatonin ELISA

The melatonin ELISA was performed as described (de Borsetti et al., 2011; Kazimi and Cahill, 1999). Triplicate samples containing 5 larvae each were homogenized in 0.5 mL 0.1 N NaOH at 6 am on night 5 and 6 pm on day 6. One mL methylene chloride (9315, J.T. Baker) was added to each sample, followed by vortexing for 30 seconds and centrifugation at 4250 g for 3 hours at 4°C. The organic phase was collected and 0.5 mL 0.1 N NaOH was added, followed by vortexing for 30 seconds and centrifugation for 20 minutes at 4250 g at 4°C. The organic phase was collected and 0.15 g sodium sulfate (SX0760, EMD Millipore) was added to each sample, followed by another 30 second vortex and 20 minute centrifugation at 4250 g at 4°C. One mL of the organic phase from each sample was transferred to a 15 mL falcon tube and wrapped in foil to protect from

light. The solution in each tube was evaporated using a rotary evaporator with a room temperature water bath. The residue was dissolved in 200 μL phosphate buffered saline (PBS) with 0.1% gelatin (214340, Becton Dickinson). Melatonin levels were assayed using a melatonin saliva ELISA kit (101720-920, Alpco Diagnostics) according to the manufacturer's instructions.

Pineal Serotonin (5-HT) Quantification

Larvae were raised in E3 medium until 6 dpf and fixed at 6 pm (day) or 6 am (night) in 4% paraformaldehyde (PFA) overnight at 4oC. Samples were washed in 0.25% Triton X-100 in PBS (PBSTx). Brains were dissected while leaving skin on the dorsal side of the brain intact to avoid removing pineal gland cells, and treated with 1 mg/ml collagenase for 45 minutes at room temperature. Samples were blocked in 2% normal goat serum/2% DMSO in PBSTx for at least two hours at room temperature. Antibody incubations were performed in blocking solution overnight at 4oC. Rabbit anti-serotonin (1:1000, S5545; Sigma) and Alexa-488 anti-rabbit (1:500, A11034, Life Technologies) antibodies were used. Samples were washed in PBSTx and mounted in 50% glycerol/PBS. Pineal imaging was performed using a confocal microscope (Zeiss 780 LSM, 40x objective, 2x digital zoom) using the same settings for all samples. ImageJ was used to create a summation z-projection for each confocal stack and the integrated pixel density for the entire pineal gland was used to quantify 5-HT fluorescence.

per1b Fluorescent In Situ Hybridization

Larvae were raised in E3 medium in either LD (lights on at 9 am and off at 11 pm) or DD (starting at 6 hpf) 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 per1b (Dekens and Whitmore, 2008) and the TSA Plus System (NEL741001, PerkinElmer). Samples were developed using Cy3 amplification reagent at 1:200 for 10 minutes and stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (268298, EMD Millipore) in 2% DMSO PBSTx for at least 4 hours at room temperature. Samples were then washed in PBSTx and mounted in 50% glycerol/PBS. Imaging was performed using a confocal microscope using the same settings for all samples (Zeiss 780 LSM, 10x and 40x objectives). 31 optical sections of 0.75 μm thickness each (23.25 μm total) were acquired for each sample. ImageJ was used to create a summation z-projection for each stack and the integrated pixel density within a region of interest of the same size for all samples was used to quantify total Cy3 fluorescence.

Quantitative PCR

Larval zebrafish were raised on either a 14:10 hour light:dark cycle at 28.5°C with lights on at 9 am and off at 11 pm, or in constant darkness. At 4 dpf, individual larvae were placed into each well of 96-well plates (7701-1651, Whatman) in dim red lighting and placed into constant darkness. Total RNA was collected using Trizol reagent (15596-026, Life Technologies) from 24 pooled larvae starting at 6 pm on day 5 every 6 hours for 24 hours. 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 an ABI PRISM 7900HT (Life Technologies) instrument. Δ Ct was calculated using ribosomal protein l13α (rpl13α) as a reference gene. Relative expression levels were plotted by determining ΔΔCt by normalizing to the LD sample or the WT LDDD sample (for Figures S3G-S3H and S4K-S4L, respectively) with the highest ΔCt value for each gene.

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Chapter 3
The role of Adenosine signaling in sleep wake behavior in zebrafish

Abstract

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.

Introduction:

The brain-energy hypothesis which proposes to explain the function of sleep posits that the purpose of sleep is to replenish the energy stores of the brain. Accordingly, an indication that there is a need of sleep may be the accumulation of lower energy molecules, specifically adenosine, the low energy metabolite of adenosine tri-phosphate (ATP), the energy currency of cells (Benington and Craig Heller, 1995). This hypothesis has been supported by research indicating that in response to sleep deprivation, adenosine accumulates in a specific part of the brain, namely the basal forebrain and cortex, in response to increased time spent in a wakeful state (Porkka-Heiskanen et al., 1997, 2000). There are 4 human and murine adenosine receptors. They include the Adora1(A1R), Adora2A (A2AR), Adora2B(A2BR) and Adora3(A3) receptors. Of these the A1R and A2AR have been shown to be involved in sleep regulation in vertebrates (Brown et al., 2012; Fredholm et al., 2001; Olah and Stiles, 2000). A1R, A2AR and A2BR are expressed in the brain (Fredholm et al., 2001; Olah and Stiles, 1992) and it is known that the A1R and A3R interact with the inhibitory G protein subunit Gi, whereas A2AR and A2B interact with the stimulatory G protein subunit. As a result, A1R and A3R cause a decrease in intercellular cAMP levels and thus the inhibition of the neurons/cells they are present in. However, A2AR and A2B cause an increase in cAMP and thus an activation of the neurons/cells they are present in (van Calker et al., 1979). Over the last 30 years, more and more specific adenosine receptor agonist and antagonists have been developed (Jacobson and Gao, 2006; Müller and Jacobson, 2011). The effect of these on sleep has been studied mostly on nocturnal rodents. The A1 agonists including N6-cyclohexyl adenosine (CHA), N6-Cyclopentyladenosine (CPA) and N6-(2-

Phenylisopropyl)adenosine (PIA) have been shown to cause an increase in sleep following treatment during their active (dark) (Alam et al., 2009; Benington et al., 1995; Blanco-Centurion et al., 2006; Methippara et al., 2005; Oishi et al., 2008) and inactive (light) (Martin et al., 1989; Radulovacki et al., 1984; Ticho and Radulovacki, 1991) phases respectively.

The A1 antagonists, including 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-Cyclopentyl-1,3-dimethylxanthine (8CPT) and 1,3-dipropyl-8-phenylxanthine (CPDX), cause an increase in wakefulness in nocturnal animals during their inactive phase (when injected into the lateral hypothalamus), but causes no change in activity during their active phase (when injected into the lateral pre-optic area) (Alam et al., 2009; Methippara et al., 2005; Thakkar et al., 2010).

The A2A agonists include CGS21680 (CGS) and 2-[(2-aminoethylamino)carbonylethyl phenylethylamino]-5-N- ethylcarboxamido adenosine (APEC). It has been previously shown that an addition of A2A agonists cause an increase in sleep and a decrease in wake behavior in rodents following drug treatment during both the active and inactive phases (Methippara et al., 2005; Satoh et al., 2006; Scammell et al., 2001). Based on these results together, it is hypothesized that A1 must be present in wake active neurons and A2A in sleep active neurons, which would result in a silencing of wake active neurons, and an activation of sleep active neurons thus pushing the brain into a sleep state after a period of prolonged wakefulness.

There is a discrepancy in results related to adenosine signaling wherein the adenosine receptor agonists and antagonists have large effects on sleep wake behavior (Alam et al., 2009; Benington et al., 1995; Satoh et al., 1998; Thakkar et al., 2010), but

adenosine receptor knock-outs show little to no change in sleep wake architecture (Huang et al., 2005; Stenberg et al., 2003). One theory is that this is because of developmental compensation in animals. This theory is supported by the result that while A1R knock-outs exhibit no changes in sleep-wake behavior, an RNAi knockdown of adenosine A1R in the basal forebrain in adult animals, results in a significant reduction in REM sleep and an increase in wakefulness similar to that seen in response to A1R antagonist injections (Alam et al., 1999; Basheer et al., 2000; Thakkar et al., 2003). We were proposing to circumvent the effects of developmental compensation by testing adenosine mutant zebrafish larvae at 4dpf.

An important question is, where in the brain does adenosine signaling act? Several brain areas associated with adenosine signaling have been identified in mammals. A1R, being inhibitory (van Calker et al., 1979; Freissmuth et al., 1991), has been hypothesized to silence wake active areas, while the A2AR, being excitatory (van Calker et al., 1979; Olah and Stiles, 1992) has been hypothesized to activate sleep centers. 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). With experiments involving injection of A1 agonists and antagonists, the basal forebrain, the lateral hypothalamus, lateral preoptic area and the prefrontal cortex have all been identified as being involved in mediating the effects of A1R on sleep (Basheer R, 2000, Methippara MM 2005, Thakkar MM 2008, Dort CJV 2009). Of these the role played by A1R in the basal forebrain has been characterized the most carefully, given that the basal forebrain is the only region in the brain to exhibit increases in adenosine level (Porkka-

Heiskanen et al., 2000). Cholinergic neurons in the basal forebrain were also thought to be involved in this process but this still remains unclear, since ablation of these neurons does not affect adenosine build up or increase the sleep amounts following sleep deprivation (Blanco-Centurion 2006, Kalinchuk AV 2008, Kalinchuk AV 2015). A2AR is expressed less widely in the brain than A1R. Higher expression levels are seen in striatum, nucleus accumbens and olfactory tubercle (Dixon et al., 1996) in rat brains. However microdialysis and injection experiments with the A2A agonists and antagonists 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). However more recent studies agonist/antagonist experiments suggest that actually the A1R and not the A2AR (or both) responsible for mediating the effects of adenosine signaling in the VLPO and the TMN (Zhang J 2013, Oishi Y 2008).

Based on the findings discussed above, we decided to use zebrafish to ask several key questions related to adenosine signaling and sleep.

1. Is adenosine signaling conserved in zebrafish?

2. Where in the brain do adenosine agonists/antagonists act to regulate sleep wake behavior?

3. Do adenosine receptor mutants exhibit changes in sleep-wake behavior and sensory responsiveness?

4. Do the adenosine agonists/antagonists affect sleep via their corresponding adenosine receptors and if so which of the homologous receptors mediate these effects?

Results

Adenosine receptor agonists and antagonists affect larval zebrafish sleep and locomotor activity.

Adenosine receptors Adora1 (A1R) and Adora2A (A2AR) are involved in sleep regulation in vertebrates. Preliminary results from a small molecule screen in zebrafish larvae showed that general adenosine receptor agonists adenosine and chloro-adenosine increased sleep while specific A1R antagonists 8-cyclopentyl-1,3- dipropylxanthine (DPCPX) and 1,3-diethyl-8- phenylxanthine (8CPT) increased activity and reduced sleep (Rihel et al., 2010). This suggests that adenosine signaling plays a role in the regulation of zebrafish sleep and A1R may be involved. To confirm these results and test whether the zebrafish A2AR is involved in sleep-wake behavior, as in mammals, we used small molecule agonists and antagonists that are specific for mammalian A1R and A2AR. To first test the function of A1R signaling, we treated zebrafish larvae with A1R agonist, (±)-5'-Chloro-5'-deoxy-ENBA (SENBA) and the A1R antagonist DPCPX. Of the A1R agonists and antagonists, SENBA is known to be one of the most specific A1R agonists (Jacobson and Gao, 2006) and DPCPX has been shown to be specific for A1R binding (Jacobson and Gao, 2006; Lohse et al., 1987) and has been used to inhibit A1R signaling in the study of sleep regulation (Gallopin et al., 2005; Thakkar et al., 2010)

To determine whether these drugs have an effect on sleep, we used a video-tracking assay (Prober et al., 2006), to compare the sleep/wake behavior of drug treated larvae to that of their DMSO vehicle treated siblings. We found that before drug treatment (day/night 5 and day 6pre), both groups of larvae exhibit similar amounts of activity and sleep (Fig. 1A-F). However, after drug addition, SENBA treated animals are

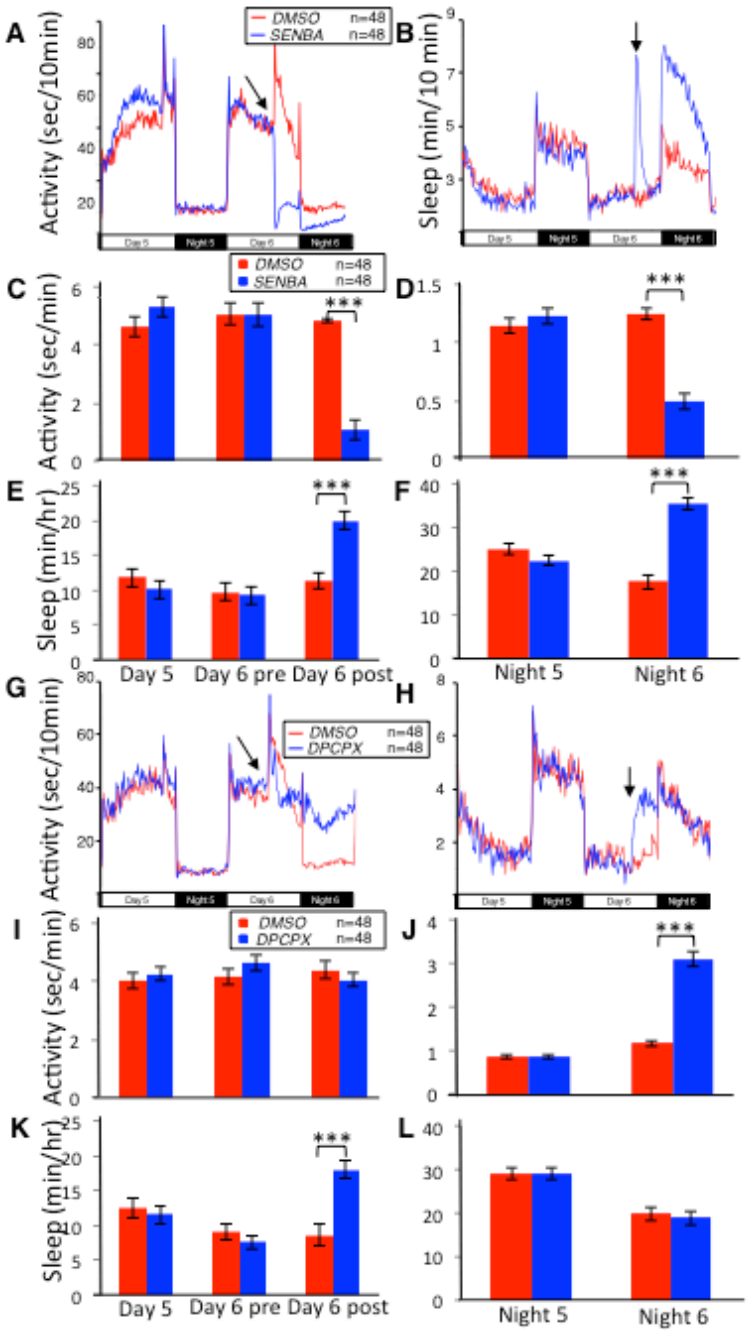

Figure 3.1: Adenosine receptor 1 (A1R) Agonists and antagonists affect locomotor activity and sleep behavior in zebrafish. Zebrafish larvae are less active and sleep more during the day (A-C, E) and night (A, B, D, E) following A1R agonist, SENBA, treatment during day 6 (as indicated by the arrows in A and B). Larvae were treated with vehicle (red) or 20uM SENBA (blue) starting on day 6. In contrast zebrafish treated with the A1R antagonist are more active during the night (G, J) and sleep more during the day (H,K) following A1R antagonist, DPCPX treatment. Larvae were treated with vehicle (red) or 20uM DPCPX (blue). Bar graphs represent mean ± SEM, n=number of larvae. ***=$p<0.0001$ compared to the vehicle treated control according to Tukey's test. Arrows indicate transient artifacts due to pipetting of drug or vehicle into the plates

less active and exhibit more sleep during both the day and night in comparison to vehicle treated larvae (Figure 3.1 C-F). There is a 75% decrease in activity and a 50% increase in sleep during both the day and night following drug treatment. The increase in sleep amount during the night is inferred to an increase in sleep bout length (mean length of sleep bouts ± SEM= 2.2 ± 0.3 vs. 2.9 ± 0.3 p=0.1 and 3.2 ±1.4 vs. 9.9 ± 0.15 p<0.0001 for day 6 post vehicle vs. drug treated larvae and Night 6 vehicle vs. drug treated larvae respectively. p-values were calculated by the unpaired t test). There is no significant difference in sleep bout number (mean number of sleep bouts ± SEM= 7.5 ± 0.5 vs. 8.3 ± 0.8; p=0.39 and 5.2 ±0.38 vs. 5.1 ± 0.37; p=0.85) for day 6 post vehicle vs. drug treated larvae and Night 6 vehicle vs. drug treated larvae respectively p-values were calculated by the unpaired t test). These results are in agreement with the observed increased sleep amounts seen in nocturnal rodents in response to A1R agonist treatment during their active (dark) (Alam et al., 2009; Benington et al., 1995; Blanco-Centurion et al., 2006; Methippara et al., 2005; Oishi et al., 2008) and inactive (light) (Martin et al., 1989; Radulovacki et al., 1984; Ticho and Radulovacki, 1991) phases respectively.

Following DPCPX addition larvae exhibit an increase in sleep during the day and an increase in activity levels during the night in comparison to vehicle treated control animals (Fig 3.1G-L). This is a 65% increase in sleep on day 6 following treatment and a 60% increase in activity on night 6 as compared to the controls (Fig 3.1J, 1K). It has been previously reported that A1R antagonists cause an increase in wakefulness in nocturnal animals during their inactive phase (when injected into the lateral hypothalamus), but causes no change in activity during their active phase (when injected into the lateral pre-optic area) (Alam et al., 2009; Methippara et al., 2005; Thakkar et al., 2010).

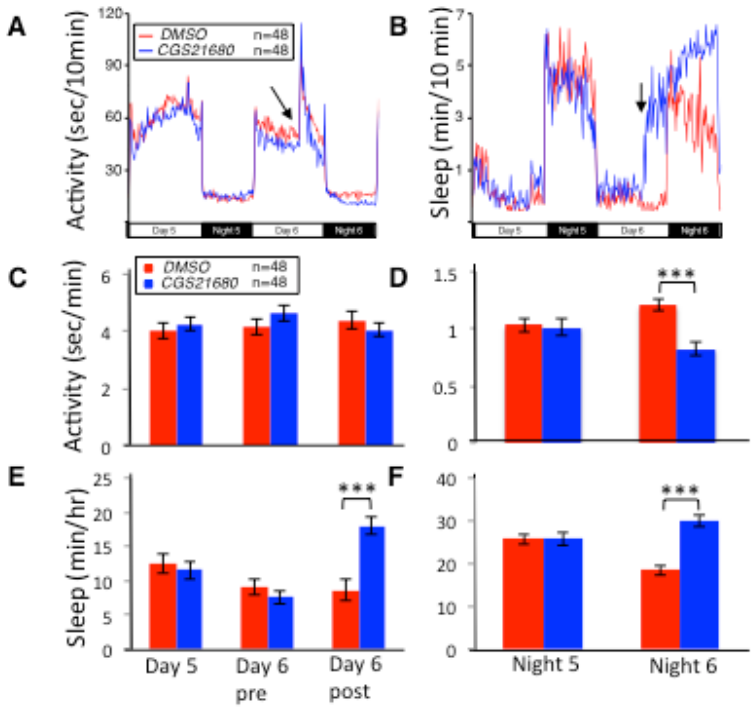

Figure 3.2: Adenosine receptor 2 (A2AR) Agonist affects locomotor activity and sleep behavior in zebrafish. Zebrafish larvae sleep more during the day (A-C,E) and night (A,B,D,F) following A2AR agonist, CGS21680, treatment. Larvae were treated with vehicle (red) or 150uM CGS21680 (blue) starting on day 6. Arrows indicate transient artifacts due to pipetting of drug or vehicle into the plates. Bar graphs represent mean ± SEM, n=number of larvae. ***=$p<0.0001$ compared to the vehicle treated control according to Tukey's test.

We next tested the effect of stimulating A2A receptors on sleep by treating larvae with the A2A agonist CGS21680 Hydrochloride (CGS) which has a strong affinity for the A2AR over other adenosine receptors (Jacobson KA 2006) and has been used extensively to stimulate A2AR activity in the study of sleep regulation ((Methippara et al., 2005; Satoh et al., 1996, 1998; Scammell et al., 2001). We observed that before drug treatment both groups of larvae have similar amounts of activity and sleep. After drug addition, there is a significant increase in sleep during both the day and night for the drug treated larvae when compared to the vehicle treated larvae (Fig 3.2A-F). There is an approximately 60 percent decrease in activity as compared to controls on night 5. There is a 50% and 40% increase in sleep on day 6 following treatment and on night 6, respectively (Fig 3.2C-F). It has been previously shown that an addition of CGS causes an increase in sleep and a decrease in wake behavior in rodents following drug treatment during both the active and inactive phases (Methippara et al., 2005; Satoh et al., 2006; Scammell et al., 2001).

To clarify, we used 20uM SENBA, 20uM DPCPX and 150uM CGS, for these experiments. We decided on these concentrations after first trying a range of concentrations. Each of these concentrations gave us a reproducible behavioral phenotype without resulting in larval lethality or precipitation of the compound added.

To summarize, the A1R and A2R agonists decrease activity and increase sleep during both the active and inactive periods. This result is in agreement with similar experiments performed in rodents ((Alam et al., 2009; Benington et al., 1995; Blanco-Centurion et al., 2006; Methippara et al., 2005; Oishi et al., 2008; Satoh et al., 2006; Scammell et al., 2001) The A1R antagonist increases activity during the night, which is

in agreement with what has been shown in rodents ((Alam et al., 2009; Thakkar et al., 2010)), however during the day, we see an increase in sleep, which has not been reported so far in rodents. It has been shown that A2AR antagonists act to increase waking activity and decrease sleep when injected into the lateral preoptic area during the active phase (Methippara et al., 2005) and when injected into the lateral ventricle during the inactive phase in rodents (Kumar et al., 2013). While preliminary results indicate that A2A antagonist 4-(2-(7-amino-2-(furan-2-yl)- [1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl) phenol (ZM241385) and chlorosteryl caffeine (CSC) increases daytime activity in zebrafish at certain concentrations, these effects could not be reproduced consistently enough for inclusion. More specific A2AR antagonists such as SCH442416 and KW6002 (Jacobson and Gao, 2006) need to be tried.

Identification of zebrafish brain regions that are activated by adenosine agonists and antagonists.

What brain areas are the effectors of Adenosine signaling? Several brain areas associated with adenosine signaling have been identified in mammals. A1R, being inhibitory (van Calker et al., 1979; Freissmuth et al., 1991) has been hypothesized to silence wake active areas, while the A2AR, being excitatory (van Calker et al., 1979; Olah and Stiles, 1992) has been hypothesized to activate sleep centers. 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). The basal forebrain, the lateral hypothalamus, lateral preoptic area and the prefrontal cortex have all

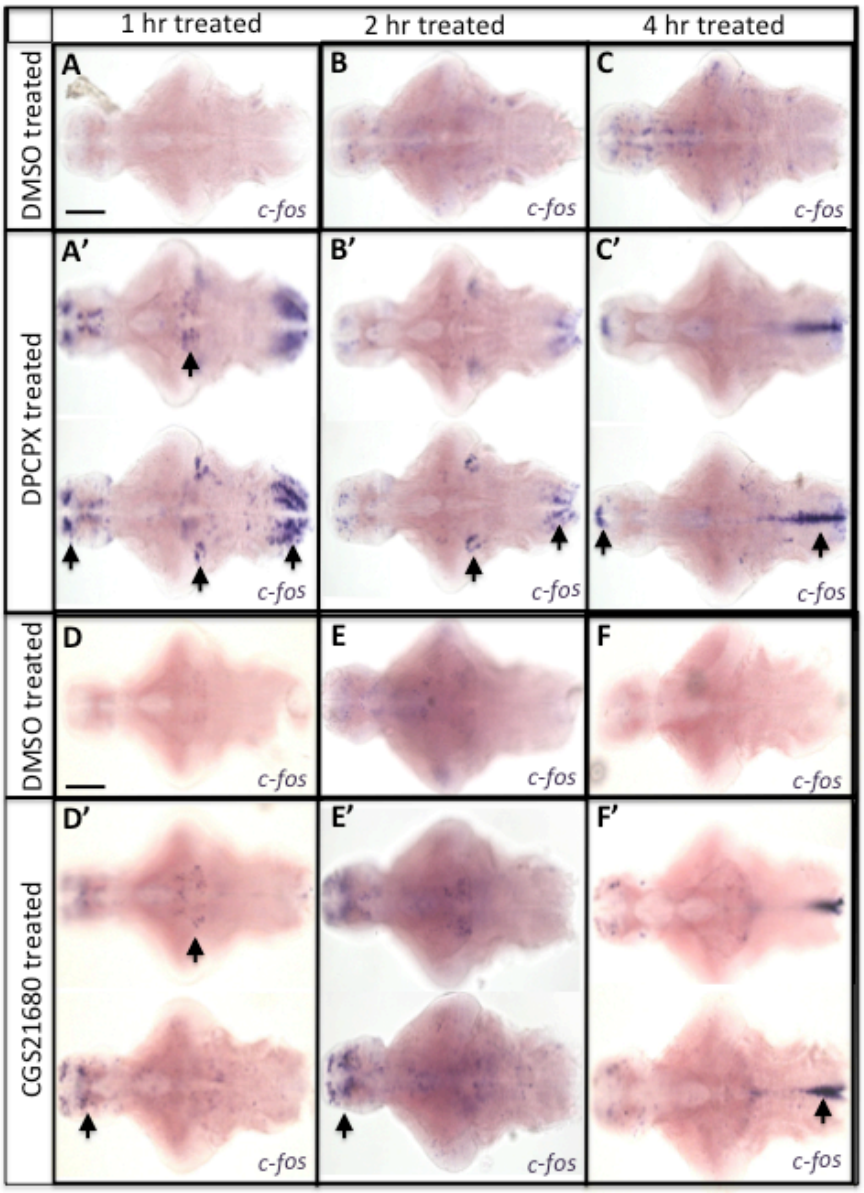

Figure 3.3: Adenosine A1R antagonist DPCPX and A2AR agonist CGS activate specific cell populations the brain: 5dpf Zebrafish larvae were treated with vehicle control (A-C, D-F)), DPCPX (A', B', C') or CGS (D', E', F') and then fixed at 1 (A,A', D,D'), 2 (B, B', E, E') or 4 hours (C,C', F,F') post treatment. Insitu hybridization was performed on dissected brains. Probe used was *c-fos*. Vehicle control animals show little to no *c-fos* labeling. DPCPX treated animals exhibit a distinctive labeling pattern in the forebrain, midbrain, hindbrain and caudal hypothalamus (see arrows). Top panels show ventral areas of the brain in focus and lower panels show more dorsal areas of the brain in focus in (A',B',C',D',E',F'). Scale bars indicate 100uM.

been identified as being involved in mediating the effects of A1R on sleep (Basheer R, 2000, Methippara MM 2005, Thakkar MM 2008, Dort CJV 2009), with experiments involving injection of A1 agonists and antagonists. Of these the role played by A1R in the basal forebrain has been characterized most carefully, since the basal forebrain is the only region in the brain to exhibit increases in adenosine level ((Porkka-Heiskanen et al., 2000). Cholinergic neurons in the basal forebrain were thought to be involved in this process but this remains unclear, since ablation of these neurons does not affect adenosine build up or the increase in sleep amounts following sleep deprivation (Blanco-Centurion 2006, Kalinchuk AV 2008, Kalinchuk AV 2015). A2AR is expressed less widely in the brain than A1R. Higher expression levels are seen in striatum, nucleus accumbens and olfactory tubercle (Dixon et al., 1996) in rat brains. However microdialysis and injection experiments with the A2A agonists and antagonists 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). However more recent studies agonist/antagonist experiments suggest it is actually the A1R and not the A2AR (or both) responsible for mediating the effects of adenosine signaling in the VLPO and the TMN (Zhang J 2013, Oishi Y 2008).

Zebrafish larvae are transparent and we can observe effects of drugs on the entire larval brain, because the blood brain barrier is being formed between 3-10dpf (Fleming et al., 2013). This allows us to visualize the areas involved in mediating the effects of adenosine signaling in the whole brain. We decided to ask which areas of the brain were involved in the mediation of the behavioral effects of the agonists and antagonists. Since

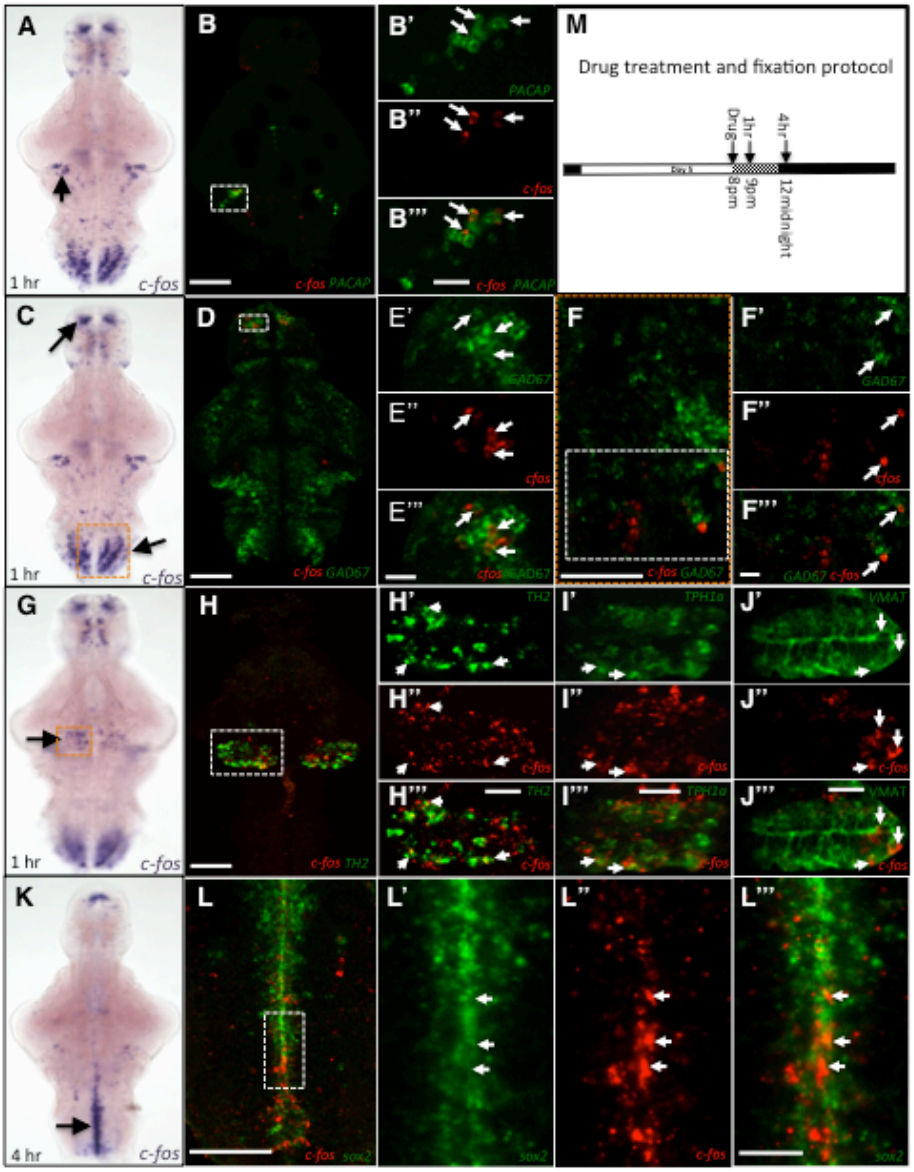

Figure 3.4: PACAP, GABAergic, Dopaminergic, Serotonergic and Sox2 positive cell populations in the brain are activated in response to the A1R antagonist. 5dpf Zebrafish larvae were treated with 20uM DPCPX and then fixed at 1 (A-J''') or 4 hours (K-L''') post treatment. Single or Double ISH was performed on dissected brains. Probes used were *c-fos* alone (A, C, G, K) or *c-fos* with *pacap* (B-B'''), gad*67* (D-F'''), *th2*(H-H'''), *tph1a*(I-I''') and *sox2*(L-L'''). Also, *c-fos* ISH with antibody staining for *VMAT*-GFP was performed (J-J'''). Rostral-hindbrain *c-fos* positive cells were *pacap* positive (B-B'''), A subset of the forebrain and hind-brain *c-fos* positive cells were positive for *gad67*(F,G), a subset of the ventral telencephalic populations were *th2, tph1a* and *vmat* positive (H-J''). Moreover the hindbrain ventricular populations were *sox2* positive (L-L'''). Arrows indicate co-locolization of the two probes in each figure. Drug treatment protocol is diagrammed in M. Scale bars =100um (B, D, F, H, L) or 20um (B'-B''', E-E''', F-F''', H'-H''', I'-I''', J'-J''', L-L'''). White boxes (in B, D, F, H and L) indicate area enlarged (in B''',D''', F''', H''' and L''' respectively). The dashed orange box in C and G indicates area of the brain imaged in F and I-I'''& J-J''' respectively.

A1 is an inhibitory receptor, presence of the A1R antagonist results in the activation of brain areas normally suppressed by A1R signaling (Thakkar et al., 2003). Since A2A is an activating G-protein coupled receptor, the presence of its agonist can be expected to in increase activity in cells expressing A2A. We therefore asked which brain regions are activated in response to inhibition of A1R by the A1R antagonist DPCPX and in response to activation of A2AR using the A2AR agonist CGS. We fixed larvae at 1 hr, 2hr and 4 hr, following addition of DPCPX or CGS, followed by in-situ hybridization (ISH) using a probe specific for the immediate early gene *c-fos,* which is used as a marker of neuronal activity (Morgan and Curran, 1991). In the case of DPCPX, we started drug treatment at 8pm and then transferred larvae to constant dark, to minimize the background activity levels seen in light. Initially we tried the same protocol at 11 am and 11pm with similar expression patterns (data not shown), we decided to continue to perform the experiment with 8pm drug treatment initiation since it was associated with the increased night-time activity phenotype. At 1 hour, 2 hours as well as 4 hour after addition of DPCPX, we observed a specific, reproducible pattern of *c-fos* expression in the brain (Fig. 3.3A'-C', 3.4A, C, G, K). At 1hr, We observed *c-fos* positive cells bilaterally in the forebrain (Figure 3.3A', 3.4C'), a prominent bilateral cluster in the rostral hindbrain close to the midbrain hindbrain boundary (Fig. 3.3A', 3.3B',3.4A), a pattern of 4 stripes in the caudal hindbrain (Fig. 3.3A', 3.4C) and bilateral clusters in the caudal hypothalamus (Fig. 3.3A',3.4G). At 2 hours we observed a subset of the cells observed at the 1hr time-point (Fig. 3.3B') , with the rostral hindbrain cluster still prominent, and less prominent caudal hindbrain and forebrain labeling. At 4 hours after drug addition a subset of brains in each sample (3/10) exhibited *c-fos* expression in ventricular cells in the hindbrain (Fig 3.3C',

3.4K). We decided to use the 1hr and 4hr time-points to identify DPCPX-activated cell populations. We performed a similar experiment with CGS, but with drug or vehicle treatment starting at 11am, a time when we knew from experiments, that CGS caused an increase in sleep and decrease in activity. At 1 hour following CGS treatment, bilateral clusters of *c-fos* positive cells in the caudal hypothalamus and bilateral clusters in the forebrain were observed (Fig. 3.3D'). At 2 hours only forebrain *c-fos* positive clusters were observed (Fig. 3.3E'). At 4 hours a subset of brains (4/10) expressed *c-fos* in the caudal hindbrain ventricular region (Fig. 3.3F'). At each of these time-points, we were careful to include a vehicle control (Fig 3.3A-F), and none of the described cell populations were observed for the corresponding vehicle control samples.

To determine the identity of the *c-fos* positive cells, we performed double fluorescent *in situ* hybridization (DFI) using a dinitro-phenyl (DNP) labeled *c-fos* probe and digioxigenin (DIG) labeled probes for *pituitary adenylate cyclase activating peptide (pacap)*, *glutamate dehydroxylase-67 (gad67), tyrosine hydroxylase 2 (th2), tryptophan-5-monooxygenase1a (tph1a) and sex determining region Y box 2 (sox2)*. In addition to these DFISH experiments, *ETvmat:GFP* (*vesicular monoamine transporter2:GFP* (*vmat-GFP*)) transgenic larva (Wen et al., 2008), which express GFP in monoaminergic neurons, were also treated with DPCPX or CGS and fixed at 1 hour post treatment to facilitate an ISH labeling with *c-fos* followed by anti-GFP immunohistochemistry, to label *vmat* positive cells. Following DPCPX treatment for 1 hour, we found that all *c-fos* positive cells in the hindbrain cluster co-localized with *pacap* (Fig. 3.4 B-B'''). The *pacap* gene is known to be involved in sleep and circadian rhythms (Mertens et al., 2007; Vaudry et al., 2009). Mutants of amnesiac, the pacap homologue in drosophila, exhibit

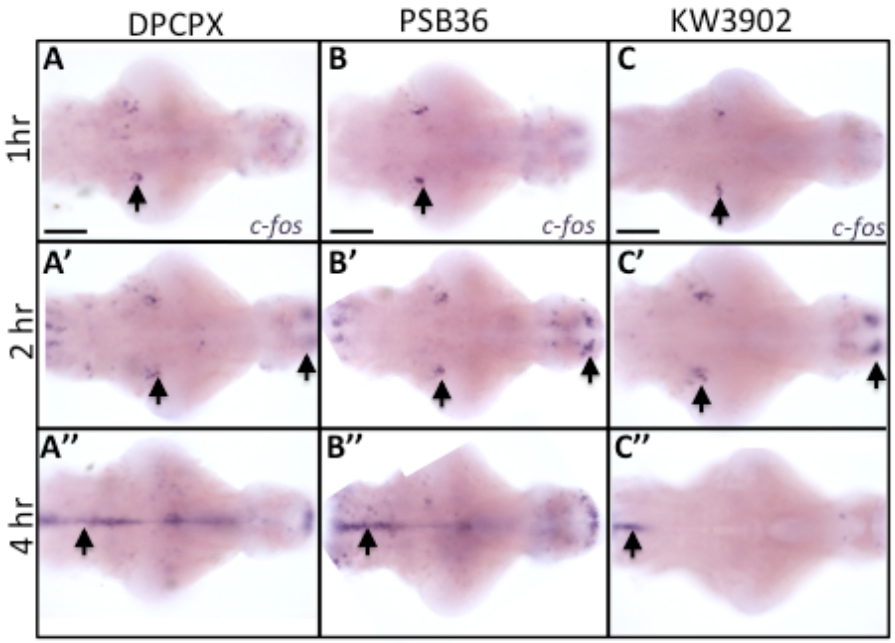

Figure 3.5: The activation pattern other A1R antagonists is very similar to DPCPX 5dpf Zebrafish larvae were treated with DPCPX (A-A''), PSB36 (B-B'') or KW3902 (C-C''), then fixed at 1 (A-C), 2 (A'-C') or 4 hours (A''-C'') post treatment. Insitu hybridization was performed on dissected brains. Probe used was *c-fos*. DPCPX treated animals exhibit a distinctive labeling pattern in the forebrain, midbrain, hindbrain (see arrows). Forebrain and hindbrain populations are not as prominent in the 1hr samples, because they were not allowed to develop for as long.

fragmented sleep bouts and defects in sleep homeostasis. We found that these hindbrain cells were not positive for the cholinergic marker *choline acetyl transferase (chat), the nitric oxide marker nitric oxide synthtase (nos1), the glutaminergic marker (vglut2a), the marker for noradrenergic cells dopamine beta hydroxylase (dbh) and gad67*. The forebrain and hindbrain *c-fos* positive cells co-localized partially with *gad67* positive cells suggesting that a subset of these *c-fos* positive cell populations were GABAergic (Fig. 3.4 E-E''', F-F'''). These cells were not positive for vglut2a or nos1. A subset of the *c-fos* positive cells in the caudal-hypothalamus co-localized with *th2*, *tph1a* and *vmat* positive cells, suggesting that they are dopaminergic and serotonergic (Fig 3.4 H-H''', I'-I''', J'-J''' respectively). Some cells in this area are negative for all 3 markers, suggesting that a population of these cells has a different identity. After 4 hours of DPCPX treatment, the hindbrain-ventricle *c-fos* positive areas co-localized with *sox2*, suggesting that these cells are ventricular ependymal cells (Fig. 3.4 L-L'''). These cells did not co-localize with the tanacyte marker *vimentin (vim)* or the glial-astrocyte marker *glial fibrillary acidic protein (gfa*p). In order to confirm that these cell populations are important for adenosine signaling, we also looked at the *c-fos* staining patterns following treatment with two other A1R antagonists, with a similar behavioral phenotypes to DPCPX (data not shown), 1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione (PSB 36) and Rollofyline (KW3902). We found that 1 hr , 2hr and 4hr treatments with these drugs resulted in a similar *c-fos* activation pattern to DPCPX (Fig. 3.5).

CGS treatment for 1, 2 and 4 hours also produced a distinctive *c-fos* activation pattern, with caudal hypothalamus and forebrain clusters seen at 1hr (Fig 3.3 D'), only

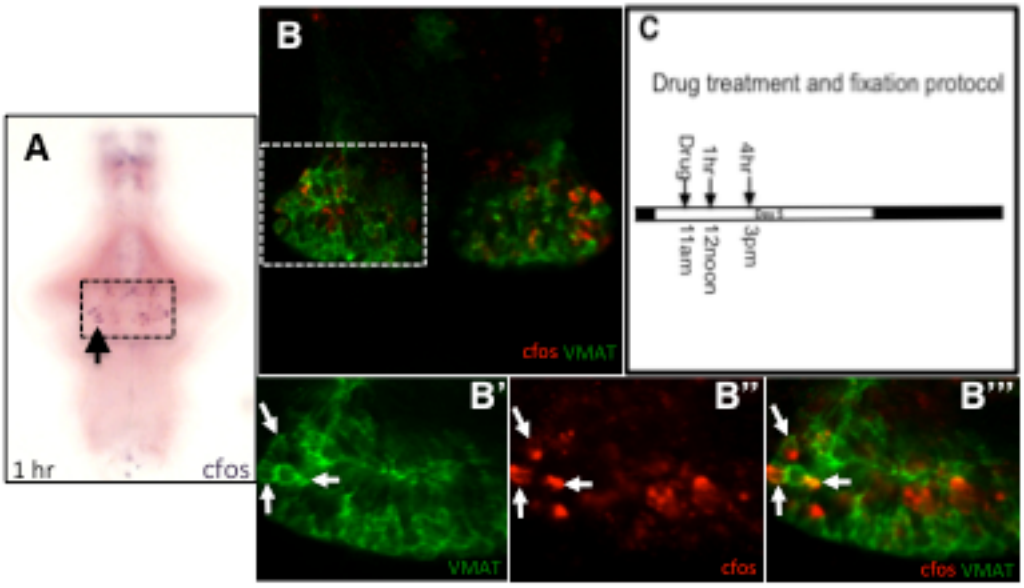

Figure 3.6: Specific VMAT positive cell populations in the brain are activated in response to the A2AR agonist. 5dpf Zebrafish larvae were treated with 20uM CGS21680 and then fixed at 1 hr post treatment (A). Single ISH or Single ISH with GFP Ab staining was performed on the dissected brains. A *c-fos* probe was used alone (A) or followed by antibody staining for *VMAT*-GFP (B-B'''). A specific reproducible pattern of *c-fos* activation was observed in the ventral telencephalon in response to drug treatment(A). A subset of these *c-fos* positive ventral telencephalic populations were VMAT positive (B-B''').

the forebrain clusters seen at 2 hours (Fig.3.3 E') and ventricular hindbrain labeling seen at 4 hours (Fig.3.3 F'). We found that the *c-fos* labeled caudal-hypothalamic cells seen at 1 hour post treatment were found to be also partially positive for VMAT (Fig 3.6 B-B'''). Suggesting that these cells may also be a mixed population of serotonergic, dopaminergic cells. The other cell populations have not yet been identified.

These results suggest an additional repertoire of cells may be activated by the adenosine agonists/antagonists, and may be part of the circuitry responsible for regulating sleep wake behavior, since their activation corresponds to the time-frame in which behavioral phenotypes of these drugs are observed. *pacap* in particular is known to be involved in sleep regulation and homeostasis (Liu et al., 2008). The co-localization results also suggest that both DPCPX and CGS may activate the same or closely positioned populations of cells in the caudal hypothalamus.

Generation of zebrafish adenosine receptor mutants

Do the adenosine receptor mutants show changes in sleep wake behavior/architecture? We next decided to generate mutants in order to be able to study the effects of the knock-outs on sleep-wake behavior, and to understand better why the effect of drug treatment yields strong behavioral effects on sleep, but the receptor knock out rodent models show normal sleep wake behavior (Huang et al., 2005; Stenberg et al., 2003). We performed a tBLASTn search of the zebrafish genome using human Adora1 protein sequence as the query. Three putative adenosine receptor sequences were identified, which we referred to as Adora1a (A1a) (ENSDARG00000075694), Adora1b (A1b)(ENSDARG00000070056) and Adora1c (A1c) (ENSDARG00000059899). These

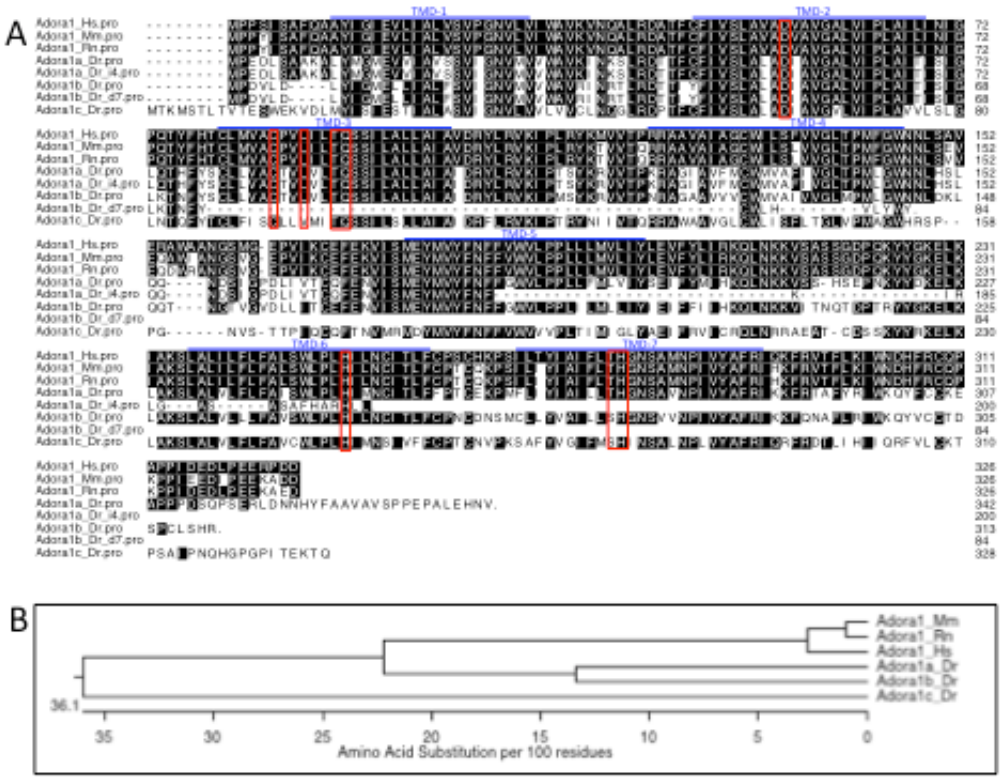

Figure 3.7: Adenosine A1 receptor and its homologs.
Zebrafish (Dr) Adora1a, 1b (A) sequence alignments with the human (Hs), mouse (Mm) and rat (Rn) Adora1 respectively using clustal W analysis are shown in the figure. The alignment also includes the Adora1a i4 and Adora1b d7mutant sequences. Conserved residues are shaded black. The 7 trans-membrane domain (TMD) regions are indicated in blue. Red boxes indicate residues determined to be important for agonist and antagonist binding for the human adenosine receptors (Fredholm et al 2001, Olah and Stiles 2000). (B) Phylogenetic tree showing evolutionary relationship between A1R paralogs.

zebrafish orthologues are well conserved with both the human and rodent receptor proteins (Fig. 3.7). Of the residues found to be important in human Adora1a for agonist and antagonist binding (Fredholm et al., 2001; Olah and Stiles, 1992) 8/8, 7/8 and 6/8 residues are conserved for zebrafish Adora1a, Adora1b and Adora1c respectively (Fig 3.7A, red boxes). This suggests that the zebrafish Adora1 receptors may show similar agonist antagonist binding properties. Using ZFN or TALEN technologies (Urnov et al., 2010, Cermak et al., 2011) we isolated a 4 bp insertion mutant for A1a and a 7 bp deletion mutant for A1b (Chen et al., 2013). Despite trying both ZFN and TALEN technologies we were unsuccessful in isolating an A1c mutant. The mutations in A1a and A1b induce a frame shift mutation at 547 bp (183rd amino acid) and 223 bp (75th amino acid) and result in truncated proteins 200 and 84 amino acids long, respectively, wherein the full protein is 342 and 313 amino acids long, respectively. It is unlikely that either of these proteins are functional because they are truncated before the C terminal domain, which is known to be important for downstream signaling and regulation of the active state in G protein coupled receptors (Palmer and Stiles, 1997).

The Adora2A receptor has been previously reported to possess two zebrafish paralogs (Boehmler et al., 2009), Adora2Aa (A2Aa) and Adora2Ab (A2Ab). These are well conserved between zebrafish and humans as well (Fig. 3.8). Of the residues important for ligand binding in the human A2A receptor, 10/10 and 9/10 residues are conserved (Fredholm et al., 2001; Olah and Stiles, 2000) (Fig. 3.8A, red boxes), suggesting similar ligand binding properties. Again, using TALEN and ZFN technologies respectively, we were able to isolate a 7 bp and 41 bp deletion mutant for A2Aa and A2Ab respectively. The mutations in A2Aa and A2Ab induce a frame shift mutation at

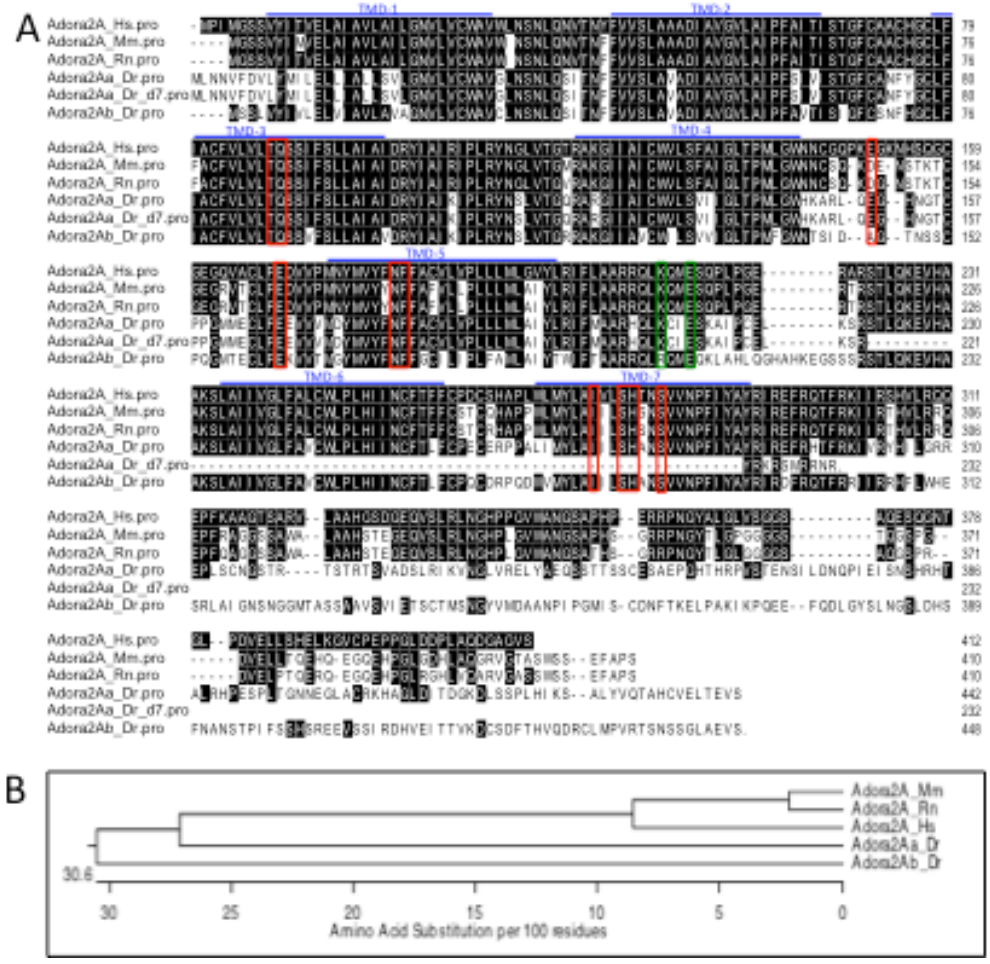

Figure 3.8: Adenosine A2A receptor and its homologs
Zebrafish (Dr) Adora2Aa, 2Ab (A) sequence alignments with the human (Hs), mouse (Mm) and rat (Rn) Adora1 respectively using clustal W analysis are shown in the figure. The alignment also includes the Adora2Aa d7 mutant sequence. Conserved residues are shaded black. The 7 trans-membrane domain (TMD) regions are indicated in blue. Red boxes indicate residues determined to be important for agonist and antagonist binding for the human adenosine receptors (Fredholm et al 2001, Olah and Stiles 2000). The green box indicates residues shown to be important for G protein binding in the case of the human A2A receptor. (Olah and Stiles 2000) (B) Phylogenetic tree showing evolutionary relationship between A2AR paralogs.

665 bp (223rd amino acid) and 400 and truncate the protein at 232 and 150 amino acids, respectively, whereas the full proteins are 443 and 448 amino acids long. It is unlikely that either of these proteins are functional because they are truncated before the C terminal intracellular domain as well as the intracellular domain between the trans membrane domains 5 and 6, which contains residues crucial important for G protein interactions of the receptor in the human protein (Fig 3.8B) (Olah and Stiles, 2000)

We attempted to perform *in situ* hybridizations for each zebrafish A1R paralog using the respective receptor mRNA sequences on 24, 36, 48 and 120 hpf larvae. However, other than weak expression of A1aR seen in the caudal hypothalamic and rostral hindbrain region (Figure 3.16), it was not possible to make any conclusions about the location of expression of zebrafish A1Rs. However, as we were able to generate probes against each of the 3 genes, from zebrafish cDNA, we conclude that each of these genes is expressed in zebrafish. The expression A2Aa and A2Ab has been described in embryos and young larvae (Boehmler et al., 2009) (Fig 3.16).

Adenosine receptor mutant larvae lack sleep phenotypes

To determine whether the zebrafish adenosine receptors are required for sleep, we used the videotracking assay described earlier. We crossed *A1a-/-;A1b-/-* and *A1a+/-;A1b+/-* adults to get sibling larvae with 4 genotypes, which we used for experiments. We found that all four genotypes exhibited comparable amounts of activity during the day and night (Fig. 3.9A, C, D). There are small but significant differences in the amount of sleep and sleep bout number between *A1a+/-;A1b+/-* and *A1a-/-;A1b-/-* larvae on Day 6,

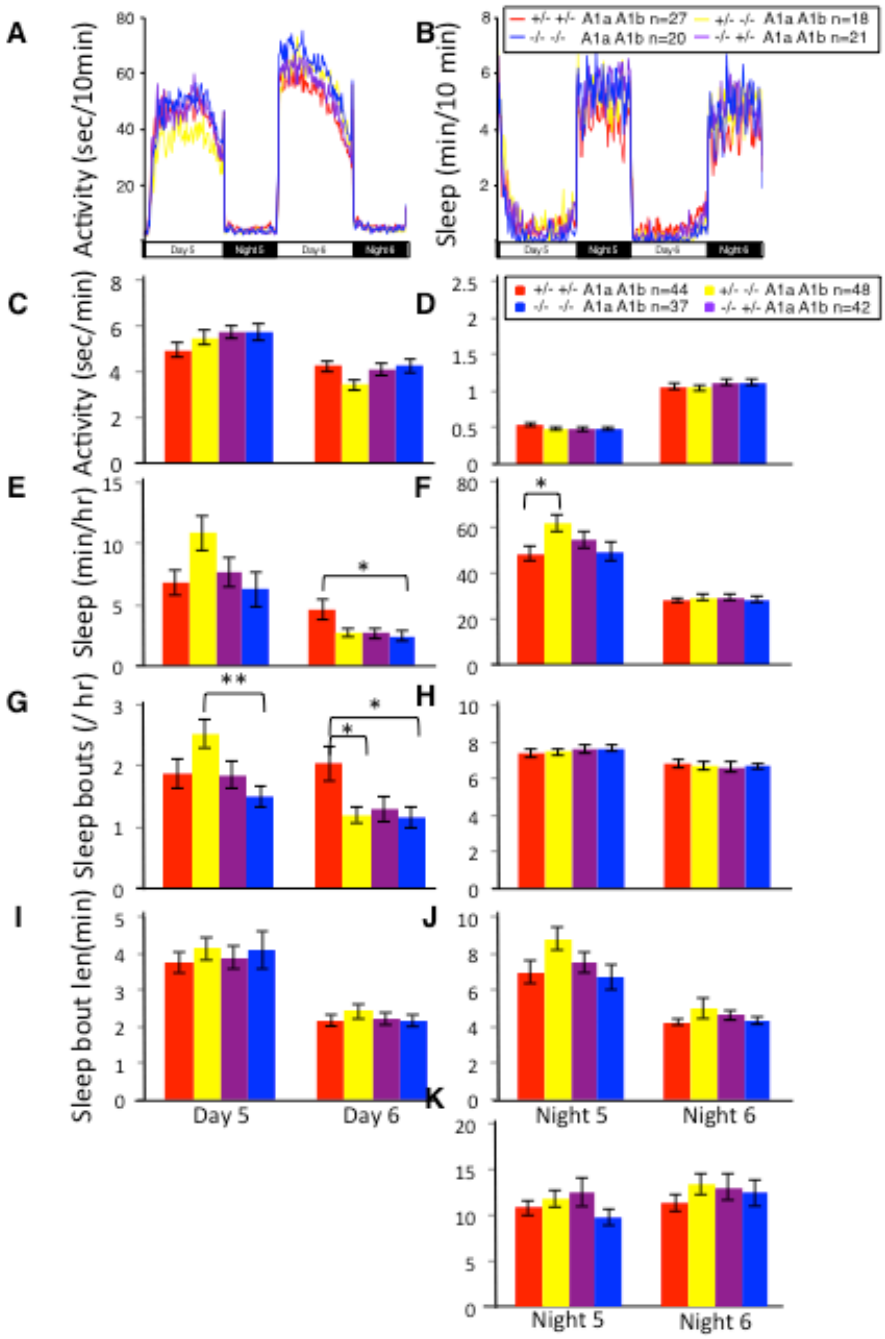

Figure 3.9: A1R mutants exhibit sleep/wake architecture similar to sibling controls A1R mutants A1a-/-;A1b-/-(blue) as well as A1a-/+;A1b-/- (yellow) and A1a-/-, A1b-/+ (purple) animals show comparable amounts of activity (A,C,D) , sleep (B,E,F), number of sleep bouts (G,H), length of sleep bouts (I,J) and sleep latency or time to first sleep after lights out (K) to A1a-/+;A1b-/+ sibling control animals (red) during both the day and night periods. Bar graphs represent mean±SEM, n=number of larvae and * =$p<0.05$; **=$p<0.01$ as compared to A1a-/+;A1b-/-+ sibling control animals in all cases according to Tukey's test.

but this difference isn't observed on Day 5 (Fig. 6B, E). More experiments would be needed to confirm this phenotype. There was no difference in sleep between the genotypes during the night (Fig. 6F). There were some differences between the sleep bout numbers between the genotypes, namely a significant decrease in sleep bout number between *A1a+/-;A1b+/-* vs. *A1a-/-;A1b-/-* and *A1a+/-;A1b-/-* again on Day 6 (Fig. 6G). But again this difference needs to be confirmed by further experimentation. There were no significant differences in the sleep bout numbers during the night between the 4 genotypes (Fig. 6H). Other aspects of sleep architecture, such as sleep bout length and sleep latency, which is the time to first sleep after lights-out at night, were comparable between the genotypes (Fig. 6I-6K).

We performed the same analysis for A2Aa and A2Ab mutants. We crossed *A2Aa-/-;A2Ab-/-* and *A2Aa+/-;A2Ab+/-* adults to get sibling larvae with 4 genotypes, which we used for experiments. We observed that all four genotypes exhibited comparable amounts of activity and sleep during the day and night (Fig. 7A-F). There were no significant differences between the genotypes in components of sleep architecture such as number of sleep bouts (Fig. 7G, 7H), length of sleep bouts and sleep latency (Fig. 7I-7K). There was however a significant difference in activity levels between day 5 and day 6 activity, but this was consistent across all genotypes, suggesting it may have something to do with the genetic background of the fish line. It will be important to out-cross these lines for future experiments and to look at day 7 activity in addition to that of day 5 and 6.

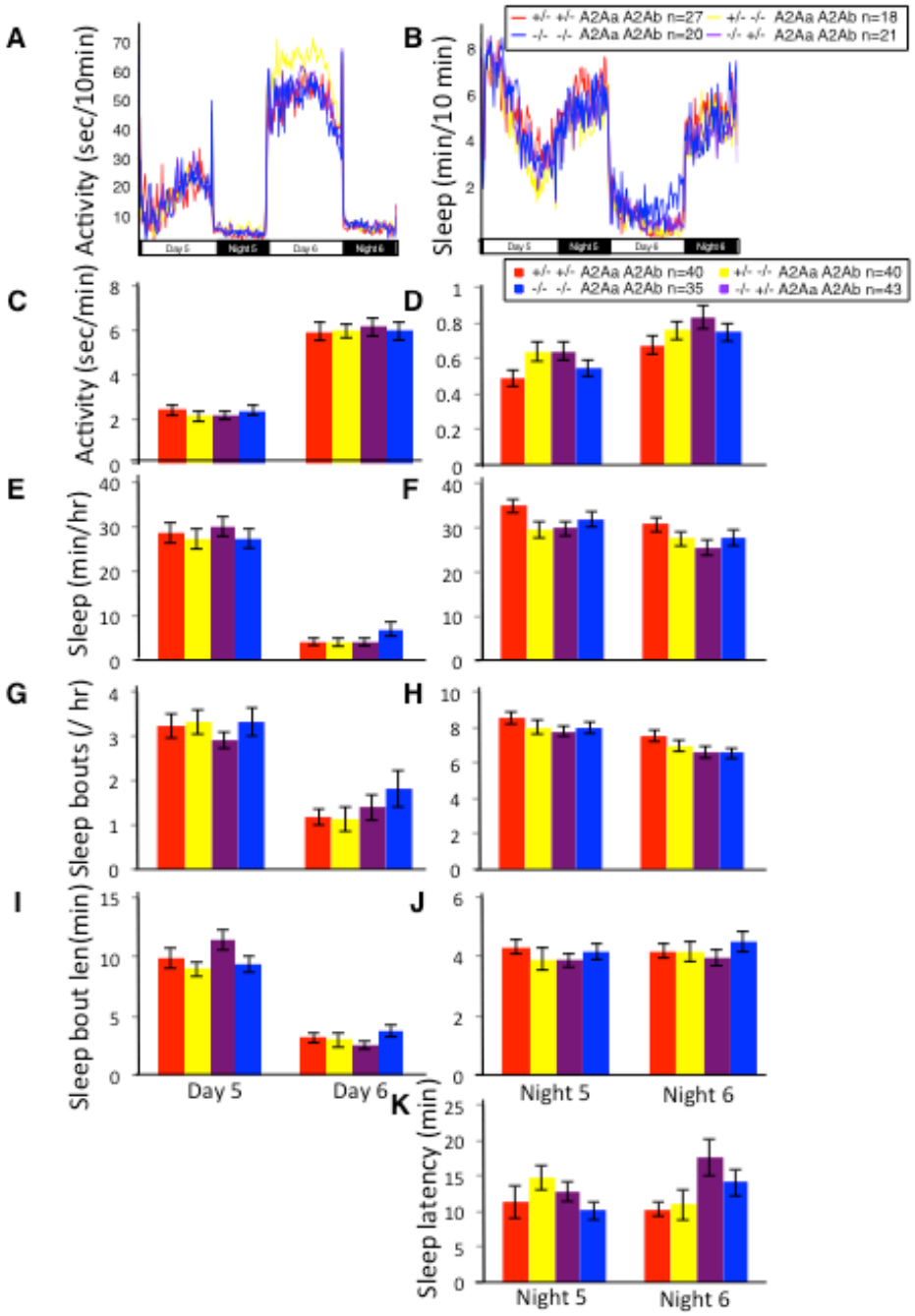

Figure 3.10: A2AR mutants exhibit sleep/wake architecture similar to sibling controls A2AR mutants A2Aa-/-;A2Ab-/-(blue) as well as A2Aa-/+;A2Ab-/- (yellow) and A2Aa-/-, A2Ab-/+ (purple) animals show comparable amounts of activity (A,C,D), sleep (B,E,F), number of sleep bouts (G,H), length of sleep bouts (I,J) and sleep latency or time to first sleep after lights out (K) to A2Aa-/+;A2Ab-/-+ sibling control animals (red) during both the day and night periods. Bar graphs represent mean±SEM, n=number of larvae and * =p<0.05; **=p<0.01 as compared to A2Aa-/+;A2Ab-/-+ sibling control animals in all cases according to Tukey's test.

These results suggest that, similar to rodents (Huang et al., 2005; Stenberg et al., 2003) the zebrafish A1 or A2 receptors alone are not required for maintenance of normal sleep-wake architecture

Testing sensory responsiveness of Adenosine receptor mutants

Sensory responsiveness of adenosine receptor mutants was tested with an arousal assay described earlier (Woods et al 2014). We hypothesized that the arousal threshold of adenosine receptor mutant larvae might be altered, since they would be expected to have reduced homeostatic sleep pressure. To test this hypothesis, we applied a mechanoacoustic stimulus (Woods et al., 2014) at night at 1 min intervals at a range of intensities. We found that *A1a-/-;A1b-/-* larvae and their sibling controls exhibited similar half-maximal response probabilities (log(probability) = 0.96±0.08, 1.013±0.07, 0.98±0.06, 1.017±0.009 for *A1a+/-;A1b+/-; A1a+/-;A1b-/- , A1a-/-;A1b+/-* and *A1a-/-;A1b-/-* respectively p = 0.92 by extra sum-of-squares F test), indicating that *A1a-/-;A1b-/-* larvae have a normal arousal threshold (Fig. 3.11A). A2Aa-/-;A2Ab-/- larvae and their sibling controls as well also exhibited similar half-maximal response probabilities. The half-maximal response probabilities (log(probability) = 1.092±0.08, 1.099±0.07, 1.077±0.08, 1.148±0.009 for *A2Aa+/-;A2Ab+/-*; *A2Aa+/-;A2Ab-/-* , *A2Aa-/-;A2Ab+/-* and *A2Aa-/-;A2Ab-/-* respectively p = 0.84 by extra sum-of-squares F test) (Fig. 3.11B). These results suggest that both A1a;A1b and A2Aa;A2Ab mutant larva show similar sensory responsiveness implying similar arousal thresholds.

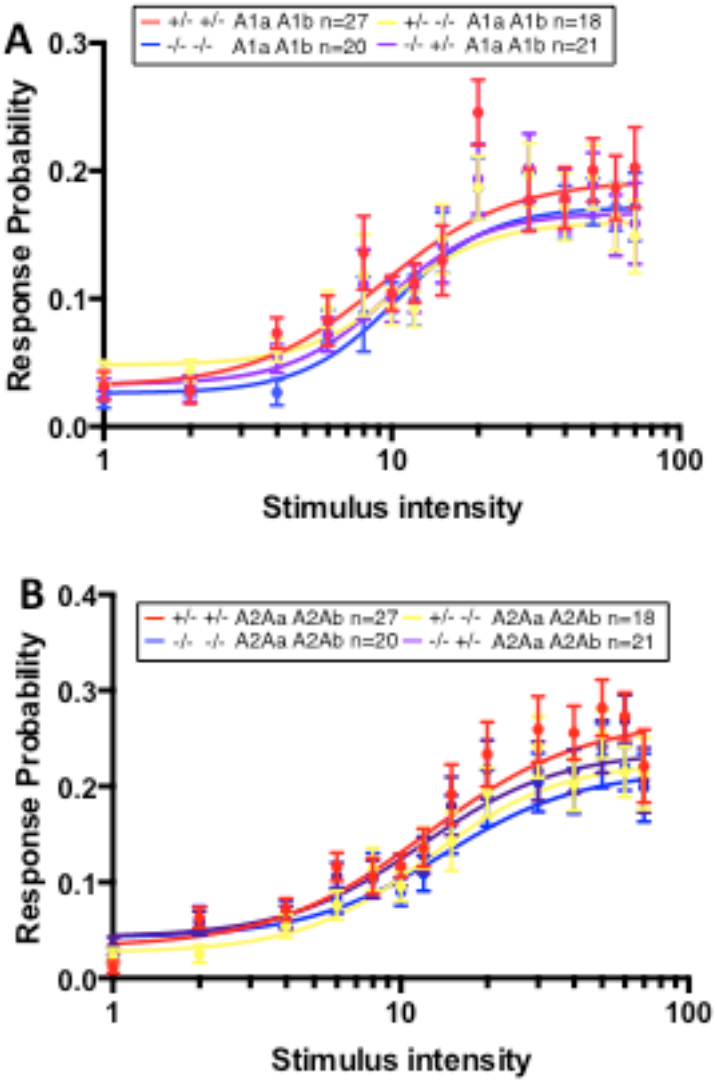

Figure 3.11: Adenosine receptor mutants exhibit similar sensory responsiveness. The response probability to a mechanoacoustic stimulus applied once per minute at night over a range of stimulus intensities is similar for all A1R mutant genotypes (log(half-maximal response probability)= 0.96±0.08, 1.013±0.07, 0.98±0.06, 1.017±0.009 for A1a-/-;A1b-/-; A1a-/+;A1b-/- , A1a-/-, A1b-/+ and A1a-/+;A1b-/+(A) It is also similar for all A2AR mutant genotypes with (log(half-maximal response probability)= 1.092±0.08, 1.099±0.07, 1.077±0.08, 1.148±0.009 for A2Aa-/-;A2Ab-/-; A2Aa-/+;A2Ab-/- , A2Aa-/-, A2Ab-/+ and A2Aa-/+;A2Ab-/-+ (B)

Sedation induced by the A1-specific agonist SENBA is abolished in A1a mutant larvae

To test the specificity of adenosine receptor ligands, we treated adenosine receptor mutants with their respective agonist and antagonists and assayed effects on sleep-wake behavior. To test whether the A1 agonist SENBA acts through A1a and A1b for its effect on sleep, we treated *A1a+/-;A1b-/- and A1a-/-;A1b-/-* siblings with 20uM SENBA or vehicle. Similar to its effects on wild type animals (Fig 1A-F), we found that SENBA reduced activity levels and increased sleep during both the day and night in *A1a+/-;A1b-/-* larvae compared to vehicle control (Fig. 3.12A-F, see red and yellow). This was similar to the effect of SENBA on wild type animals. However, the effect of SENBA was abolished in *A1a-/-;A1b-/-* larvae (Fig. 3.12A-F, see purple and blue). To determine whether loss of A1a or A1b alone is sufficient to block the effects of SENBA, we performed this experiment in the *A1a* mutant background and treated *A1a-/-;A1b+/- and A1a-/-;A1b-/-* siblings with 20uM SENBA or vehicle (Fig 3.13A-F). We found that there was no effect of SENBA in either genotype. This result indicates that in zebrafish, A1aR but not A1bR is required for the behavioral effects of the A1R agonist SENBA.

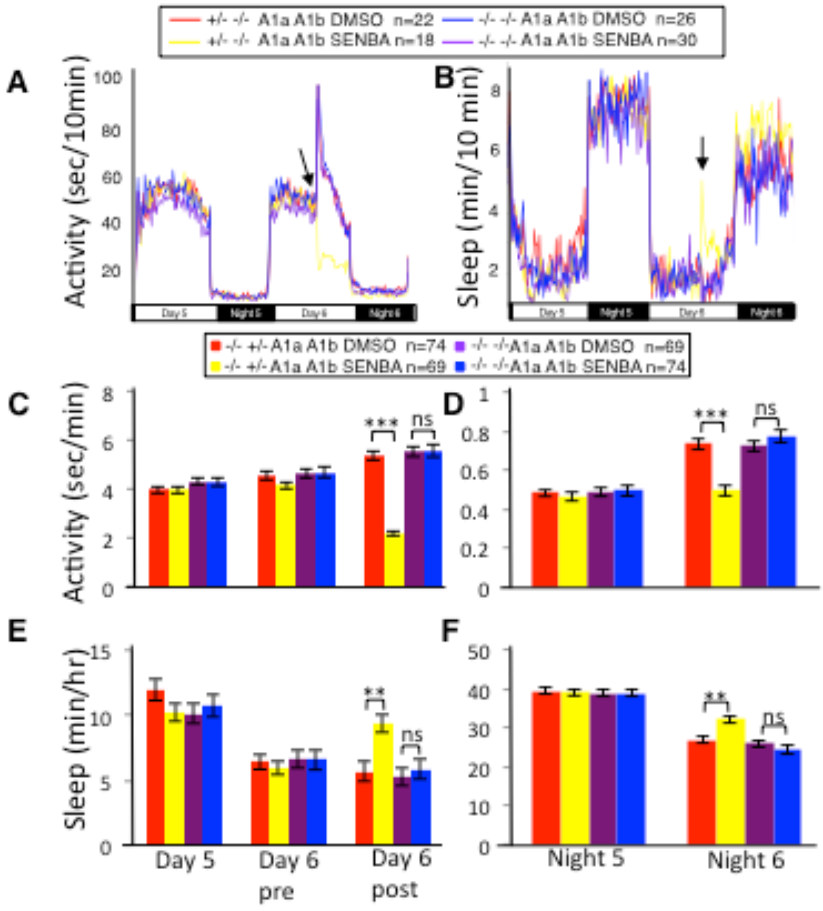

Figure 3.12: The zfA1aR is sufficient for the behavioral effect of theA1R agonist
Larvae were treated with DMSO vehicle or 20uM SENBA on day 6. After drug treatment, on both day and night 6, A1a-/+;A1b-/- animals display reduced activity and increased sleep as compared to vehicle treated animals (A-F, see yellow and red respectively). In contrast, after drug treatment, 20uM SENBA treated A1a-/-;A1b-/- animals display similar amounts of activity and sleep as the respective vehicle treated animals (A-F, see blue and purple respectively). Bar graphs represent mean±SEM, n=number of larvae and *** =p<0.0001; as compared to the indicated vehicle treated control animals in all cases as measured by Tukey's test.

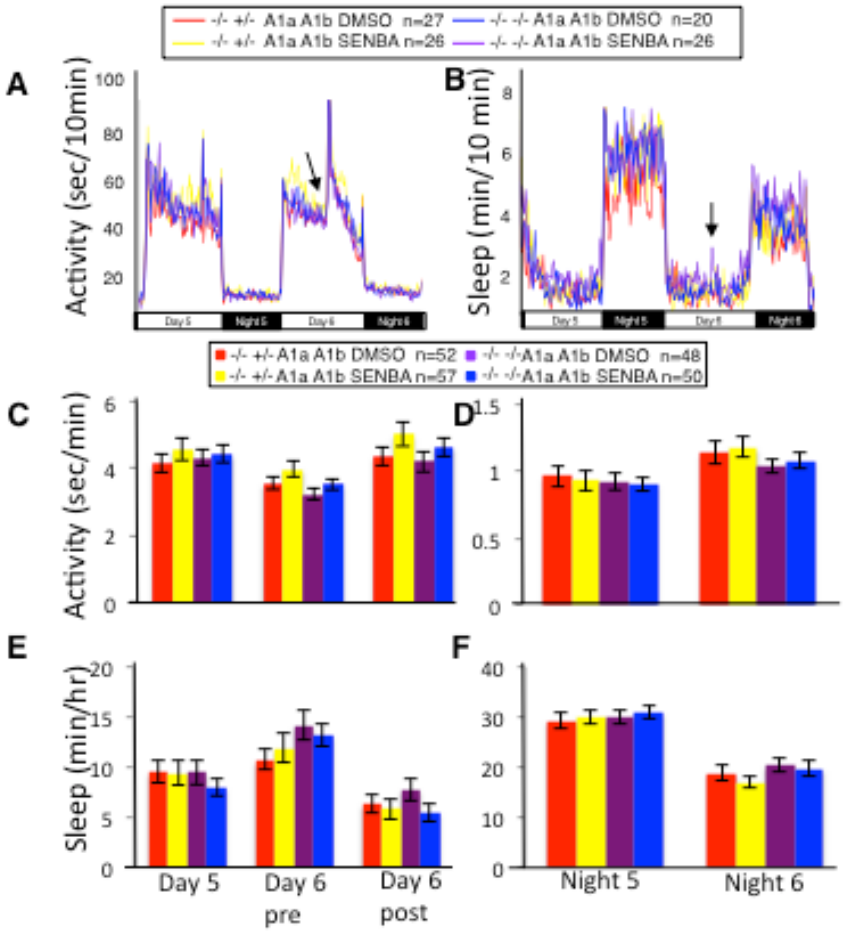

Figure 3.13: The zfA1aR is sufficient for the behavioral effect of theA1R agonist (A1a mutant background)

Larvae were treated with DMSO vehicle or 20uM SENBA on day 6. After drug treatment, on both day and night 6, A1a-/-;A1b+/- as well A1a-/-;A1b-/- as animals display no significant change in activity or sleep as compared to vehicle treated animals (Tukey's test performed). Bar graphs represent mean±SEM, n=number of larvae.

Arousal induced by the A1-specific antagonist DPCPX is not abolished in A1a; A1b double mutant larvae

To test whether the A1 antagonist DPCPX acts through A1a and A1b for its effect on sleep, we treated *A1a-/-;A1b+/- and A1a-/-;A1b-/-* siblings with 20uM DPCPX or vehicle. Similar to its effects on wild type animals (Fig 1A-F), we found that DPCPX increased sleep during the day and increased activity levels during the night in *A1a+/-;A1b-/-* larvae compared to vehicle control (Fig. 3.14A-F, see red and yellow). However unlike, SENBA, there was a similar effect on DPCPX on the in *A1a-/-;A1b-/-* larvae (Fig. 3.14, see purple and blue). These results suggest that the A1aR and A1bR are not is required to mediate the behavioral effects of the DPCPX antagonist.

Sedation induced by the A2a-specific agonist CGS is not abolished in A2Aa; A2Ab double mutant larvae

To test whether the A2A agonist CGS requires A2Aa and A2Ab to promote sleep, we treated *A2Aa+/-;A2Ab-/- and A2Aa-/-;A2Ab-/-* siblings with vehicle or CGS. Similar to its effects on wild type animals (Fig 2A-F), we found that CGS increased sleep and decreased activity during the day and night in *A2Aa+/-;A2Ab-/-* larvae compared to vehicle control (Fig. 3.15A-F, see red and yellow). There was however, a similar effect of CGS on the *A2Aa-/-;A2Ab-/-* larvae (Fig. 3.15, see purple and blue). These results suggest that the A1aR and A1bR are not is required to mediate the behavioral effects of the A2A agonist.

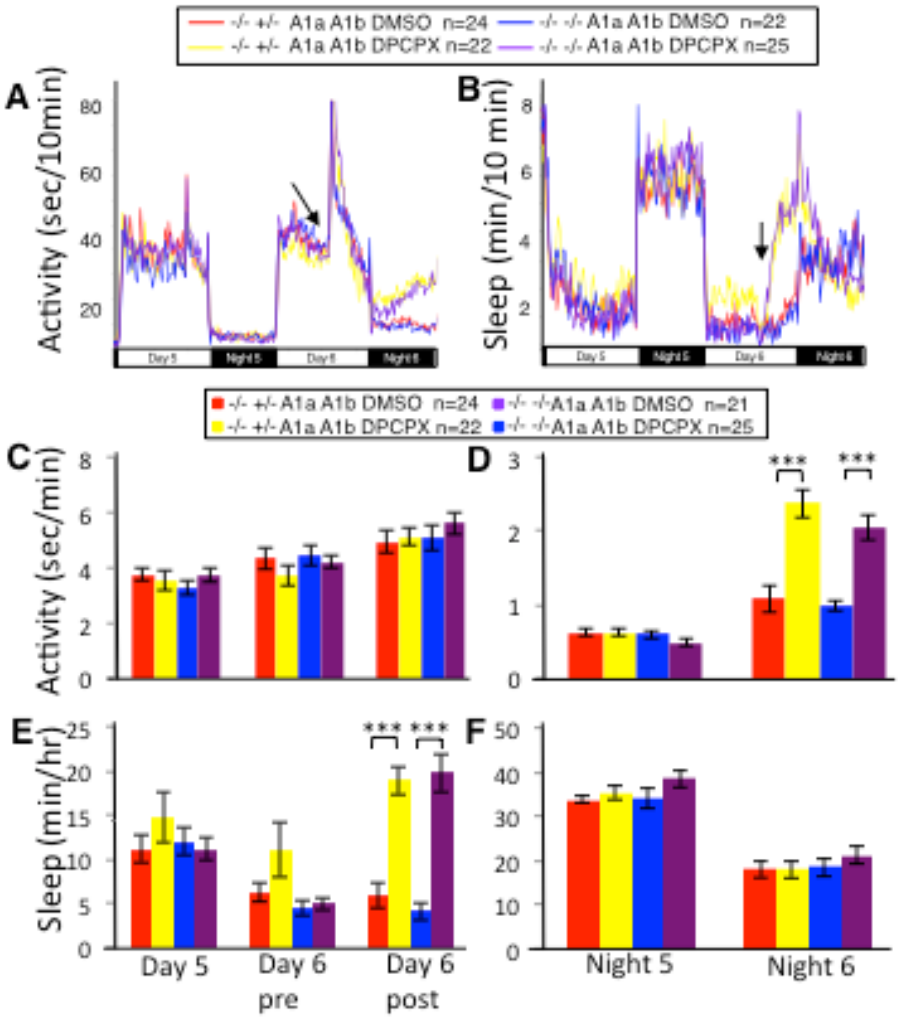

Figure 3.14: The zfA1aR and zfA1bR are not sufficient to mediate the effects of the A1R antagonist. Larvae were treated with DMSO vehicle or 20uM DPCPX on day 6. After drug treatment, on both day and night 6, A1a-/-;A1b+/- and A1a-/-;A1b-/- animals display increased sleep during the day and increased activity at night in comparison to their respective vehicle treated controls (A-F, see yellow and red vs purple and blue respectively). Bar graphs represent mean±SEM, n=number of larvae and *=p<0.05; **=p<0.001; *** =p<0.0001; as compared to the indicated vehicle treated control animals in all cases as measured by Tukey's test.

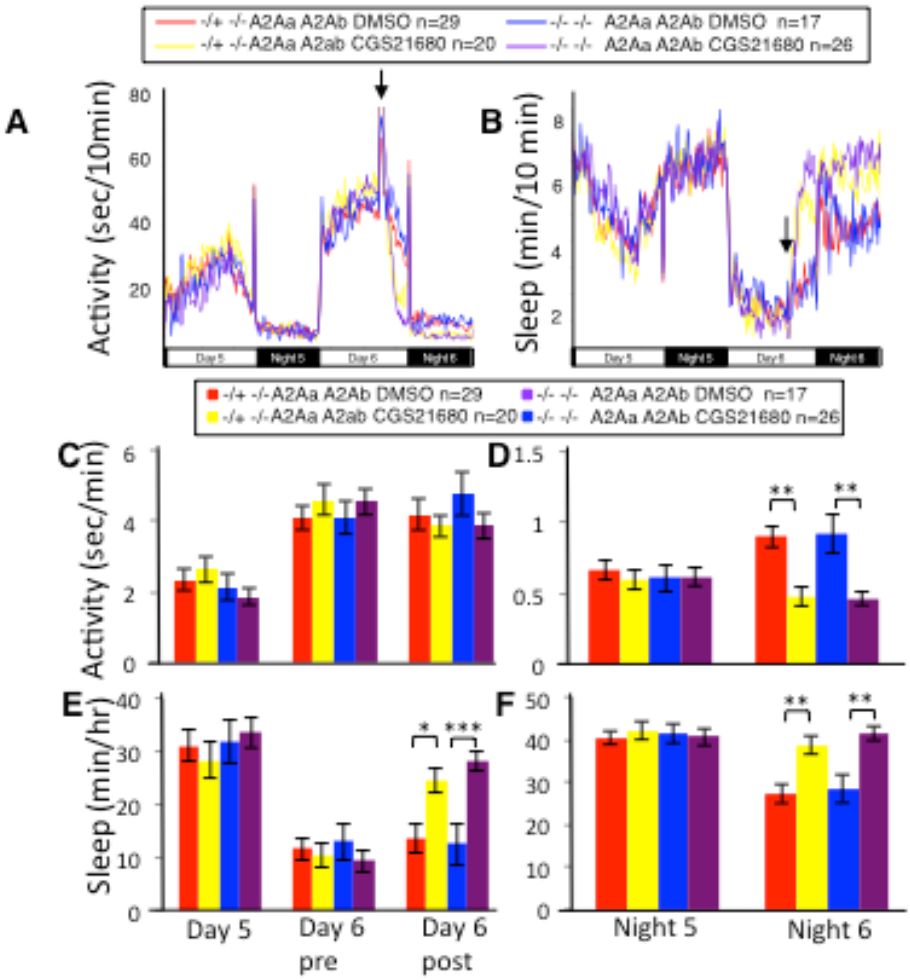

Figure 3.15: The zfA2AaR and zfA2AbR are not sufficient to mediate the effects of the A2AR agonist. Larvae were treated with DMSO vehicle or 150uM CGS21680 on day 6. After drug treatment, on day6 post treatment and night 6, A2Aa-/+;A2Ab-/- and A2Aa-/-;A2Ab-/- larvae sleep more during the day and night in comparison to their respective vehicle treated controls (A-F, see yellow and red vs. purple and blue respectively). Bar graphs represent mean±SEM, n=number of larvae and *=p<0.05; **=p<0.001; *** =p<0.0001; as compared to the indicated vehicle treated control animals in all cases as measured by Tukey's test.

The results suggest that DPCPX and CGS maybe acting through other adenosine receptors, given that both DPCPX and CGS have an affinity for A2B and A3 receptors respectively as well (Jacobson and Gao, 2006). It is also possible that the A1c receptor, for which we have not been successful in generating mutants, may play a role in mediating the DPCPX behavioral phenotype . We, however also have to consider the possibility that perhaps these two drugs have off-target effects. It should however be noted that, in spite of the ambiguity in how these drugs mediate their behavioral phenotype, they still appear to target areas of the brain that control sleep-wake behavior, suggesting that the neural-population identification *c-fos* results are interesting and important.

Discussion:

Our study examined the role played by adenosine signaling in the regulation of larval zebrafish sleep-wake behavior. Based on preliminary results from an earlier drug screen, (Rihel et al., 2010), we hypothesized that adenosine signaling and the A1R may specifically play a role in zebrafish sleep-wake behavior. We tested this hypothesis and found that the A1R agonist/antagonist and the A2AR agonist affects zebrafish sleep-wake behavior. The A1R agonist SENBA decreases activity and increases sleep during the day and night following drug treatment, with respect to vehicle controls. These results are in agreement with the observed increased sleep amounts seen in nocturnal rodents in response to A1R agonist treatment during their active (dark) (Alam et al., 2009; Benington et al., 1995; Blanco-Centurion et al., 2006; Methippara et al., 2005; Oishi et al., 2008) and inactive (light) phases (Martin et al., 1989; Radulovacki et al., 1984; Ticho and Radulovacki, 1991) respectively. We tested DPCPX as well as several other A1R antagonists including PSB36 and KW3902. We observed that the A1R antagonists caused an increase in activity during the night and an increase in sleep during the day (data not shown).

A1R antagonist studies have been carried out by generally injecting the A1R antagonist into specific brain areas theorized to be involved in sleep wake behavior. It has been previously reported that A1R antagonists cause an increase in wakefulness in nocturnal animals during their inactive phase (when injected into the lateral hypothalamus). This is supported our results in zebrafish. However, it has been reported that there is no change in activity during the active phase when A1R antagonists are injected into the lateral pre-optic area (Alam et al., 2009; Methippara et al., 2005;

Thakkar et al., 2010), which is not supported by our results in zebrafish. This discrepancy between the rodent data and the zebrafish results could be because in the case of zebrafish, the whole brain is affected, and perhaps the lateral pre-optic area is not involved in A1R mediated signaling. We find that the A2A agonist CGS decreases activity and increases sleep during both the day and night following drug addition. This result is in agreement with studies in rodents, showing that an addition of A2A agonists causes an increase in sleep and a decrease in wake behavior during both the active and inactive phases (Methippara et al., 2005; Satoh et al., 2006; Scammell et al., 2001).

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. Higher expression levels are seen in striatum, nucleus accumbens and olfactory tubercle (Dixon et al., 1996) in rat brains. With experiments involving injection of A1 agonists and antagonists, the basal forebrain, the lateral hypothalamus, lateral preoptic area and the prefrontal cortex have all been identified as being involved in mediating the effects of A1R on sleep (Basheer R, 2000, Methippara MM 2005, Thakkar MM 2008, Dort CJV 2009). Microdialysis and injection experiments with the A2A agonists and antagonists 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). Based on this literature, it's evident that the identified areas involving the effects of adenosine signaling on sleep-wake behavior are in no way exhaustive. In order to locate areas of the brain involved in Adenosine

signaling in zebrafish, we used experiments with drug-treatment, followed by *c-fos* and brain marker insitu approaches. Zebrafish offered an advantage in the possibility of studying the effects of the agonist/antagonists on the whole brain. Using this approach we were able to find new populations of cells involved in mediating the effects of the agonist/antagonists on sleep-wake behavior. DPCPX activated cells including *pacap* cells in the hindbrain. The *pacap* belongs to the *vasoactive intestinal peptide* (*vip*) family of peptides. It has been reported to cause an increase in REM sleep in rodents during their active phase and have no effect on sleep wake behavior during their inactive phase (Fang et al., 1995). Its homologous to *amnesiac* (*amn*) in fruit flies wherein mutants for amnesiac have been reported to exhibit fragmented sleep and reduced amounts of recovery sleep following sleep deprivation (Liu et al., 2008). This suggests pacap plays an important role in sleep and sleep homeostasis. It would be interesting to explore further this link between DPCPX and pacap by testing the effect of DPCPX on pacap mutants. Other cell populations activated include dopaminergic and serotonergic populations in the caudal hypothalamus and gabaergic populations in the forebrain and hindbrain.

For A2AR agonist CGS, the caudal hypothalamus seems to be involved in mediating its effects on sleep-wake behavior. It is a little unusual that both A1 and A2A seem to activate the caudal hypothalamus populations. However it can't be ruled out that each drug activates a closely situated but distinct population of cells.

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). However, it is not clear whether all of these paralogs are functional. We know that at least the Adora1a gene seems to be functional as an adenosine receptor, since we are able to observe a faint insitu staining pattern for this gene (Fig 3.16 A, A') and observe that in the absence of this gene, the A1R agonist phenotype is abrogated (Fig 3.12, 3.13). We have not yet been able to establish or rule out the role/requirement of Adora1b and Adora1c in adenosine signaling in zebrafish sleep. We also have not been able to establish or rule out the role/requirement of Adora2Aa and Adora2Ab in adenosine signaling in zebrafish sleep. There is some weak evidence to suggest, however, that Adora2Aa and Adora2Ab are expressed in the zebrafish brain from in-situ results (Boehmler et al., 2009) (Fig. 3.16). Its possible that more sensitive in-situ techniques will shed some new light on this issue (Choi et al., 2014).

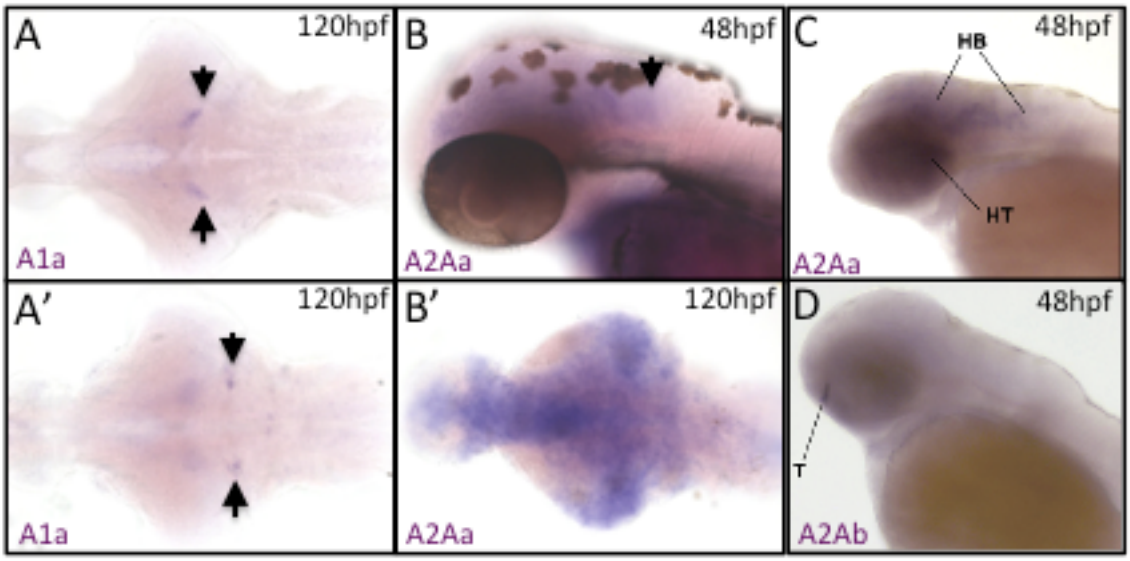

Figure 3.16: A1a, A2Aa and A2Ab expression in the zebrafish brain.
Insitu hybridization was performed with antisense probes against A1a and A2Aa. Faint A1a expression was observed in the rostral hindbrain and caudal hypothalamus area (A, A'). Faint hindbrain expression was detected in the hindbrain at 48hpf (B) and (C), but in dissected brains from 120hpf larvae, there was either diffuse A2A expression all over the brain, or the signal to noise ratio of the probe was not high enough to detect any cell clusters (B'). The only brain expression of A2Ab, was reported to be in the thalamus. But we were unable to reproduce this result. Figures (C,D) have been adapted from (Boehmler et. al., 2009).

There is a difference in results related to adenosine signaling wherein the adenosine receptor agonists and antagonists have large effects on sleep wake behavior (Alam et al., 2009; Benington et al., 1995; Satoh et al., 1998; Thakkar et al., 2010), but adenosine receptor knock-outs show little to no change in sleep wake architecture (Huang et al., 2005; Stenberg et al., 2003). One theory is that this is because of developmental compensation in animals. This theory is supported by the result that while A1R knock-outs exhibit no changes in sleep-wake behavior, an RNAi knockdown of adenosine A1R in the basal forebrain in adult animals, results in a significant reduction in REM sleep and an increase in wakefulness similar to that seen in response to A1R antagonist injections (Alam et al., 1999; Basheer et al., 2000; Thakkar et al., 2003). We hoped to circumvent the effects of developmental compensation by testing adenosine mutant zebrafish larvae at 4dpf. However, our mutant behavioral analysis suggests that there is little to no effect on sleep architecture of the adenosine receptor mutants. This may be because developmental compensation affects the sleep-wake circuits in mutant larvae at 4dpf, or that developmental compensation doesn't explain the discrepancy. It's possible that in the absence of adenosine receptors, other circuits control sleep wake architecture. It is also possible that a defect in sleep homeostasis may be observed in the mutants only in sleep deprivation conditions. It may be important to establish this through a sleep deprivation assay to test this hypothesis. It's also possible that the other adenosine receptors compensate for the lack of one receptor subtype. It would be interesting to generate a quadruple knock out, and eventually to study mutants of the A2B and A3 receptors. We observed that adenosine A1aR is required to mediate the effects of the A1 agonist on

sleep-wake behavior. This suggests that at least the zebrafish A1aR is involved in the regulation of sleep-wake behavior and is a functional adenosine receptor.

Finally, the finding that DPCPX does not require the same adenosine receptor, which is sufficient to mediate the effects of the agonist is unexpected. In the case of A1R mutant mice, the only phenotype observed was an abrogation of the effect adenosine antagonist 8CPT on delta power during NREM sleep. We cannot rule out that perhaps all three zebrafish A1 receptors need to be knocked out in order to cancel the effect of the antagonist. It is also possible that one of the other adenosine receptors, perhaps the A2B receptor, which has an affinity for DPCPX (Jacobson and Gao, 2006), mediates the effects of DPCPX. However we must also consider the possibility that antagonist has off target effects. The specificity of DPCPX for A1R was described first using the human A1R in cell culture (Lohse et al., 1987). Further it has been used to determine A1R localization in the mouse brain (Weber et al., 1990) and was used to show the decrease/absence of A1R binding sites in the brain in the A1R knock-out animals (Stenberg et al., 2003). This result suggests that DPCPX is specific for the mouse A1R, although no one seems to have tested the behavioral phenotype of DPCPX in A1R knock-out animals. Residues shown to be important for DPCPX binding in human receptors are almost entirely conserved in zebrafish (Barbhaiya et al., 1996; Olah and Stiles, 2000). DPCPX has been reported to function as a *phosphodiesterase (PDE4)* inhibitor as well (Ukena et al., 1993). To test the possibility that the behavioral effects of DPCPX are because the PDE4 inhibitory characteristics, we tested the potent PDE4 inhibitor Rollipram on larval zebrafish. This drug induced a large increase in daytime activity levels with no effect on sleep (data not shown). When we tested *c-fos* activation patterns

following Rollipram treatment, none of the previously described DPCPX populations of cells were observed to be activated (data not shown). This leaves us with the possibility of DPCPX acting through Adora1c or one of the other Adenosine receptors, such as A2B, for its effects on behavior.

The finding that CGS21680 does not act through A2Aa and A2Ab receptors is also unexpected, considering that the residues required for CGS binding (Olah and Stiles, 2000) are conserved in zebrafish A2Aa and A2Ab receptors. We also observed that the behavioral phenotype on addition of CGS is conserved between zebrafish and rodents. It has been shown in mice that in A2A receptor knock-outs the behavioral phenotype mediated by CGS and caffeine is abrogated (Huang et al., 2005; Urade et al., 2003). However, non-A2A binding sites in the brain for CGS21680 have been described in mice (El Yacoubi et al., 2000; Halldner et al., 2004). CGS21680 has also been reported to bind to A1R in the mouse hippocampus (Halldner et al., 2004). It may also be relevant to consider the possibility that CGS binds to another A2AR paralog in zebrafish.

On the whole, the study has made some relevant contributions in the examination and understanding of sleep-wake regulation in zebrafish in the context of adenosine mutants and A1R/A2AR agonists and antagonists.

Materials and methods:

Zebrafish Genetics

Adora1a, Adora1b, Adora2Aa and Adora2Ab mutant zebrafish were generated using the Zinc Finger Nuclease (ZFN) or TAL effector nuclease (TALEN) method as described (Chen et al., 2013; Reyon et al., 2012; Sander et al., 2010, 2011a) using plasmids obtained from Addgene. For Adora1a, a 4bp insertion mutant was isolated. For Adora1b, Adora2Aa and Adora2Ab, 7bp, 7bp and 41bp deletion mutants respectively were isolated. Adora1a mutants were genotyped using the primers 5'-AGCCTGCAACAGAACGACTC-3' and 5'- ACGAGCATGAAAAGCAGAGG-3'. Mutant and WT PCR products (86 bp and 82 bp, respectively) were distinguished by running the PCR reaction on a 4% agarose gel. Adora1b mutants were genotyped using the primers 5'- GGCCATTACCATAAGCATCG-3' and 5'-TCTCAGATAGCGGTCAATAGCA-3'. Mutant and WT PCR products (123 bp and 130 bp, respectively) were distinguished by running the PCR reaction on a 4% agarose gel. Adora2Aa mutants were genotyped using the primers 5'-AAGCCATCCCATGTGAACTC-3' and 5'- TCACATTCAGGGCAGAACAG-3'. Mutant and WT PCR products (144 bp and 151 bp, respectively) were distinguished by running the PCR reaction on a 4% agarose gel. Adora2Ab mutants were genotyped using the primers 5'- CCACAGCATACTTCCTTTCCA-3' and 5'-CCTTGAGGACAGGAGCTGTT-3'. Mutant and WT PCR products (161 bp and 202 bp, respectively) were distinguished by running the PCR reaction on a 2% agarose gel.

Behavioral Analysis

Larval zebrafish were raised on a 14:10 hour light:dark cycle at 28.5OC 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 of E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4) (Prober et al., 2006). In 4 genotype experiments, plates were sealed with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. The sealing process introduces air bubbles in some wells, which are discarded from analysis. 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 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 28.5OC. The parameters used for detection were: detection threshold, 15; burst, 25; freeze, 3; bin size, 60

seconds. Larvae were exposed to 20 μM (±)-5'-Chloro-5'-deoxy-ENBA (SENBA) (3576, Tocris Bioscience), 20 μM 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (0439, Tocris Bioscience) by adding 100 μL of a 130 μM stock solution to E3 embryo medium during the experiment. Larvae were exposed to 150 μM CGS21680 (1063, Tocris bioscience) by adding 100 μL of a 975 μM to E3 embryo medium. The same concentration of DMSO was used as vehicle control for each drug experiment.

Arousal Threshold Assay

The videotracking system was modified with an Arduino (http://www.arduino.cc/) based automated driver to control a solenoid (28P-I-12, Guardian Electric) delivering a tap to a 96-well plate containing larvae. Taps were applied from 12:30 am to 7:30 am the 5th night of development with an inter-trial-interval of 1 minute to measure overall arousal threshold or 5 minutes to assay arousal during sleep. Previous studies showed that a 30 second interval between repetitive stimuli is sufficient to prevent behavioral habituation (Woods et al., 2014). In experiments where stimulus intensities were varied, 14 different intensities were applied in a random manner, with 30 trials at each intensity. The response of larvae to the stimuli was monitored using the videotracking software and subsequently analyzed in Matlab (version R2013a, TheMathworks, Inc) and Excel (Microsoft). Statistical analysis was performed using the Variable Slope log(dose) response curve fitting module of Prism (Graphpad).

Data analysis

Videotracker sleep/wake data was processed using custom PERL and Matlab (version R2013a, The Mathworks, Inc) scripts. Any one minute period with no movement was defined as one minute of sleep (Prober et al., 2006). A sleep bout was defined as a continuous string of sleep minutes. Sleep latency was defined as the length of time from lights on or off to the start of the first sleep bout. Average activity was defined as the average amount of detected activity in seconds/minute, including all rest bouts. These parameters were calculated for each experimental day and night. Luminescence and

behavioral data was processed for circadian analysis using custom Matlab scripts. Raw data were fit to a damped cosine curve as described (Hirota et al., 2008). Statistical tests were performed using Prism (Graphpad).

Fluorescent and Chromogenic In Situ Hybridization

Larvae were raised in E3 medium in LD until 5 dpf. For DPCPX treatments larvae were treated with 20uM DPCPX or DMSO at 8pm and then shifted to a dark incubator till the respective timepoint of 1, 2 or 4hr. For CGS21680, sample were treated with 150uM CGS or DMSO till the respective timepoint of 1, 2 or 4hr. Samples were then quickly transferred to an eppendorf tube and fixed in 4% PFA overnight at room temperature. Chromogenic in situ hybridization was performed using published protocols (Thisse and Thisse, 2008) on dissected brains using a DIG-labeled antisense riboprobe specific for *c-fos*. Fluorescent insitu hybridization was performed with a DNP-labelled antisense riboprobe for *c-fos* and DIG-labelled antisense probes for *pituitary adenylate cyclase activating peptide (pacap)*, *glutamate dehydroxylase-67 (gad67)* (Higashijima et al., 2004)*, tyrosine hydroxylase 2 (th2), tryptophan-5-monooxygenase1a (tph1a) and sex determining region Y box 2 (sox2), (choline acetyl transferase (chat), nitric oxid synthtase1 (nos1), dopamine beta hydroxylase (dbh), vimentin (vim), vglut2a or glial glial fibrillary acidic protein acid protein (gfap)* using the TSA Plus System (NEL741001, PerkinElmer). Samples were developed using Cy3 amplification reagent at concentrations ranging from 1:100 to 1:300 for 2.5 to 30 minutes Samples were then washed in PBSTx and mounted in 50% glycerol/PBS. Imaging was performed using a confocal microscope (Zeiss 780 LSM, 10x and 40x objectives).

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Chapter 4

Conclusions

Conclusions

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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