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

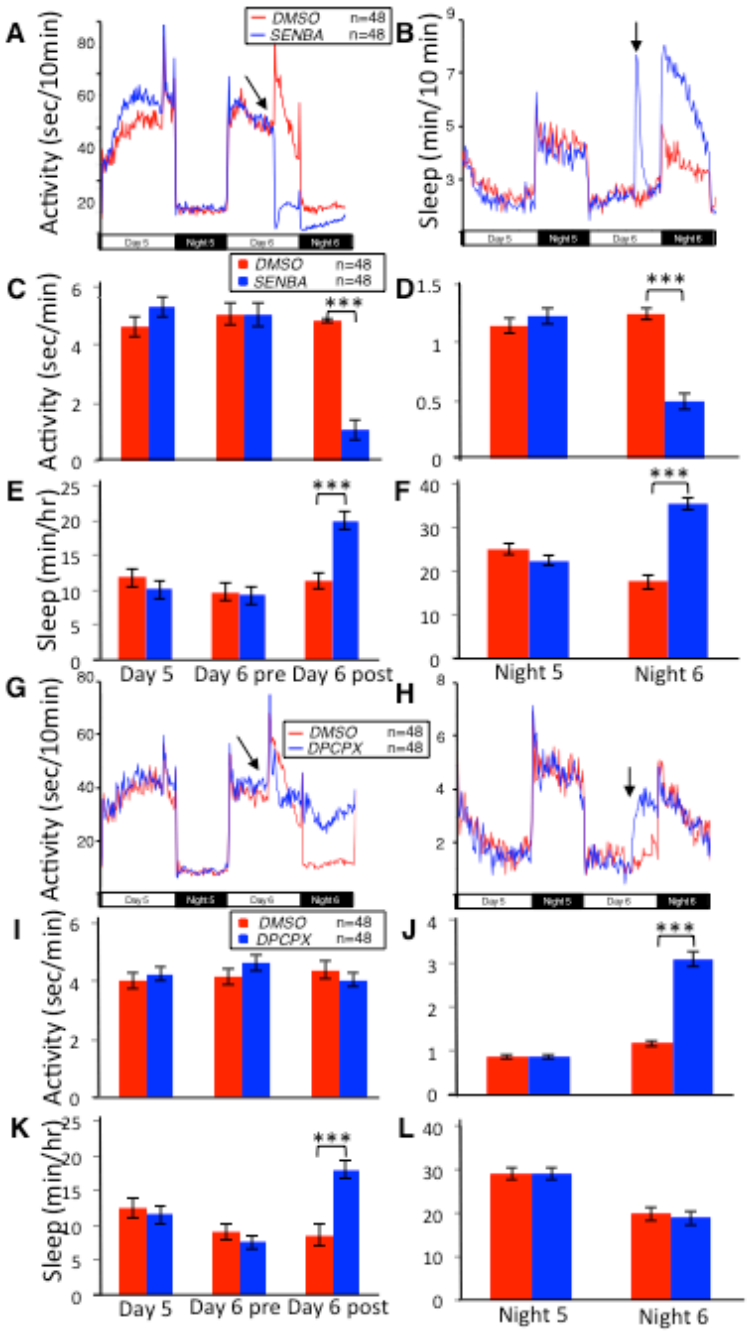

Figure 3.1: Adenosine receptor 1 (A1R) Agonists and antagonists affect locomotor activity and sleep behavior in zebrafish. Zebrafish larvae are less active and sleep more during the day (A-C, E) and night (A, B, D, E) following A1R agonist, SENBA, treatment during day 6 (as indicated by the arrows in 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).

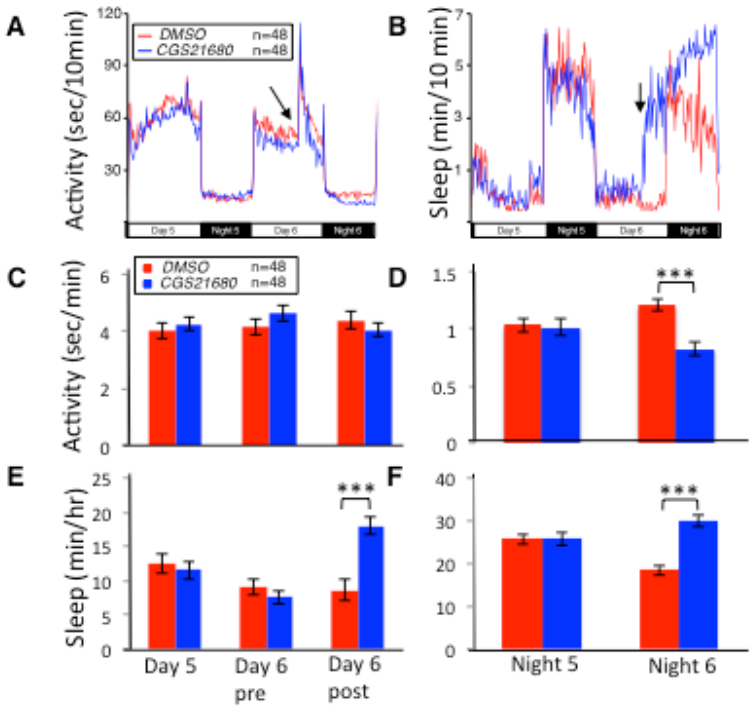

Figure 3.2: Adenosine receptor 2 (A2AR) Agonist affects locomotor activity and sleep behavior in zebrafish. Zebrafish larvae sleep more during the day (A-C,E) and night (A,B,D,F) following A2AR agonist, CGS21680, treatment. Larvae were treated with vehicle (red) or 150uM CGS21680 (blue) starting on day 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

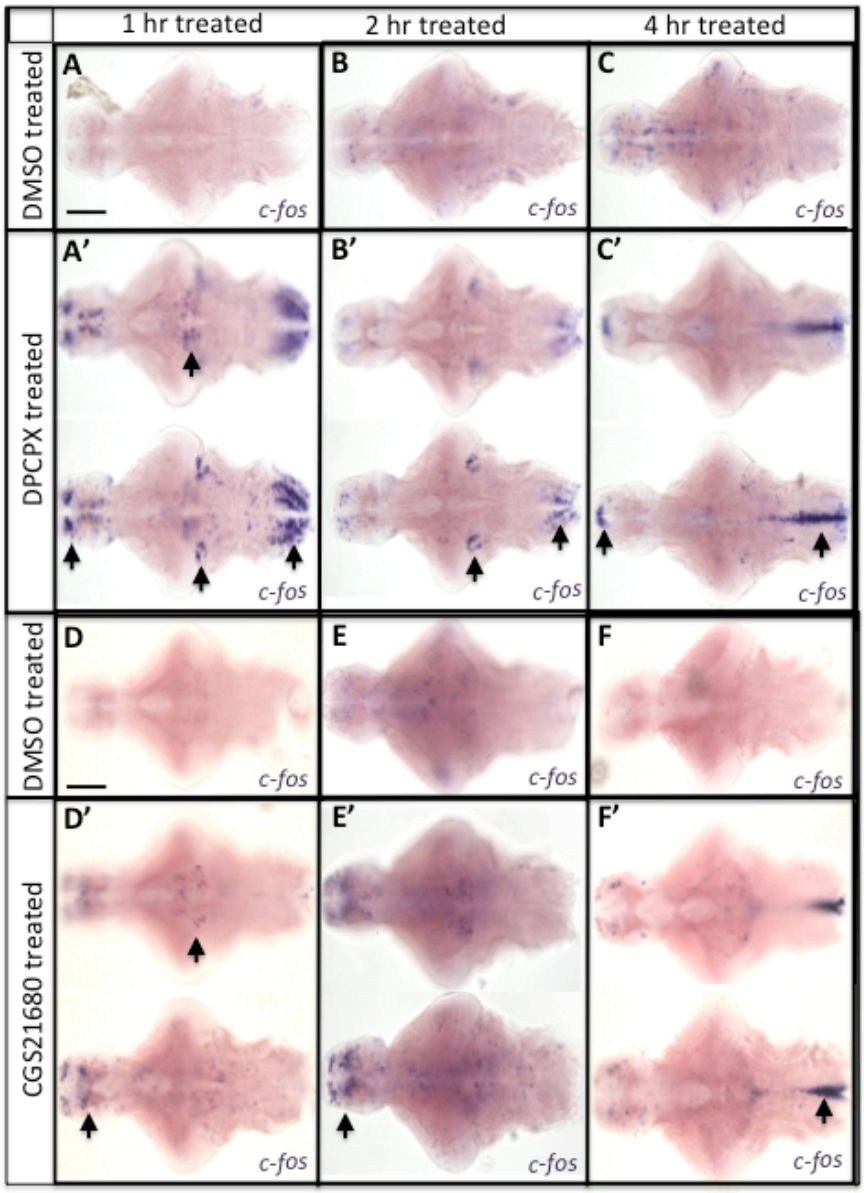

Figure 3.3: Adenosine A1R antagonist DPCPX and A2AR agonist CGS activate specific cell populations the brain: 5dpf Zebrafish larvae were treated with vehicle control (A-C, D-F)), DPCPX (A', B', C') or CGS (D', E', F') and then fixed at 1 (A,A', D,D'), 2 (B, B', E, E') or 4 hours (C,C', F,F') post treatment. 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

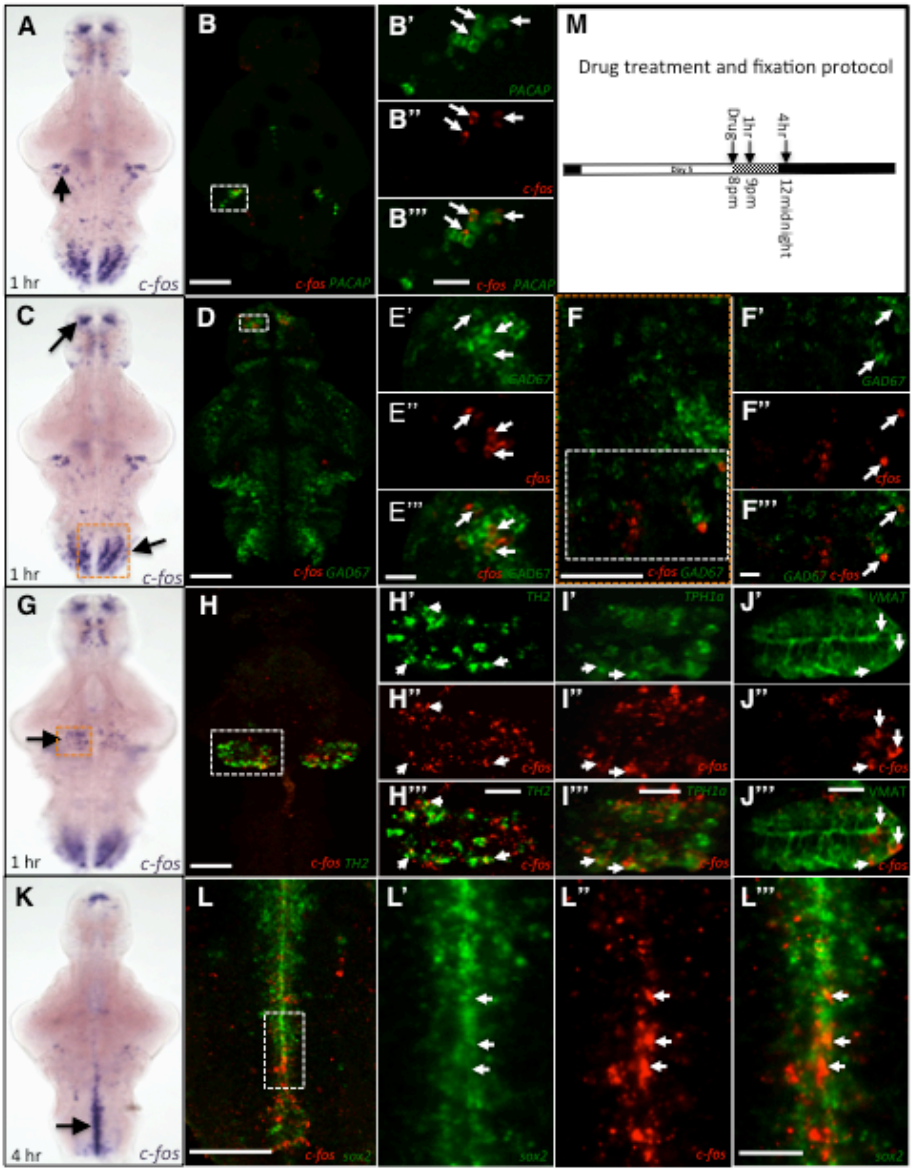

Figure 3.4: PACAP, GABAergic, Dopaminergic, Serotonergic and Sox2 positive cell populations in the brain are activated in response to the A1R antagonist. 5dpf Zebrafish larvae were treated with 20uM DPCPX and then fixed at 1 (A-J''') or 4 hours (K-L''') post treatment. Single or Double ISH was performed 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

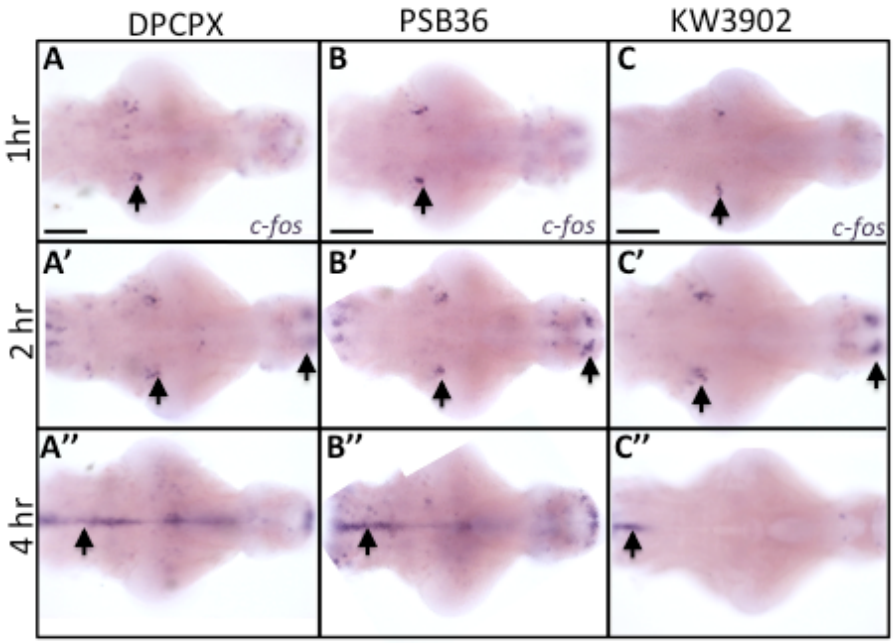

Figure 3.5: The activation pattern other A1R antagonists is very similar to DPCPX 5dpf Zebrafish larvae were treated with DPCPX (A-A''), PSB36 (B-B'') or KW3902 (C-C''), then fixed at 1 (A-C), 2 (A'-C') or 4 hours (A''-C'') post treatment. Insitu hybridization was performed on dissected brains. Probe 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

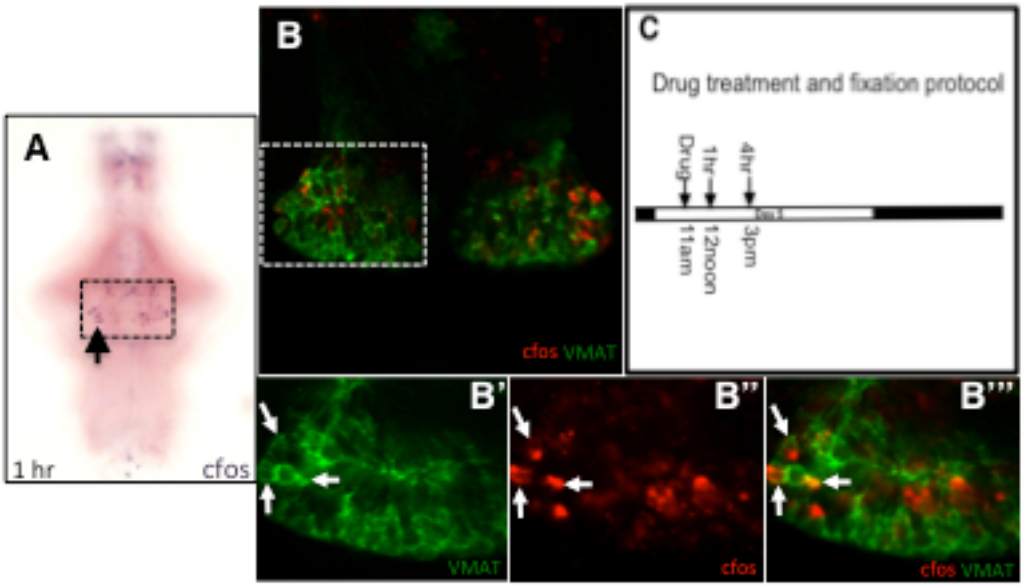

Figure 3.6: Specific VMAT positive cell populations in the brain are activated in response to the A2AR agonist. 5dpf Zebrafish larvae were treated with 20uM CGS21680 and then fixed at 1 hr post treatment (A). Single ISH or Single ISH with GFP Ab staining was performed on the dissected brains. A *c-fos* 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

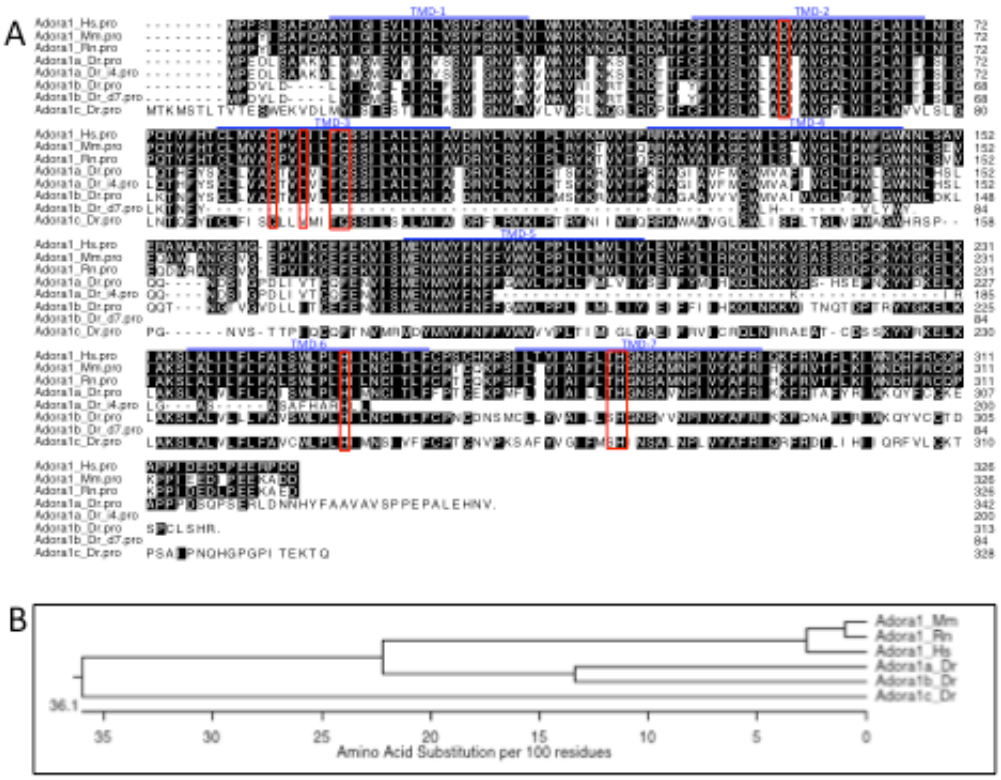

Figure 3.7: Adenosine A1 receptor and its homologs.
Zebrafish (Dr) Adora1a, 1b (A) sequence alignments with the human (Hs), mouse (Mm) and rat (Rn) Adora1 respectively using clustal W analysis are shown in the figure. The alignment also includes the Adora1a i4 and Adora1b d7mutant sequences. Conserved 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

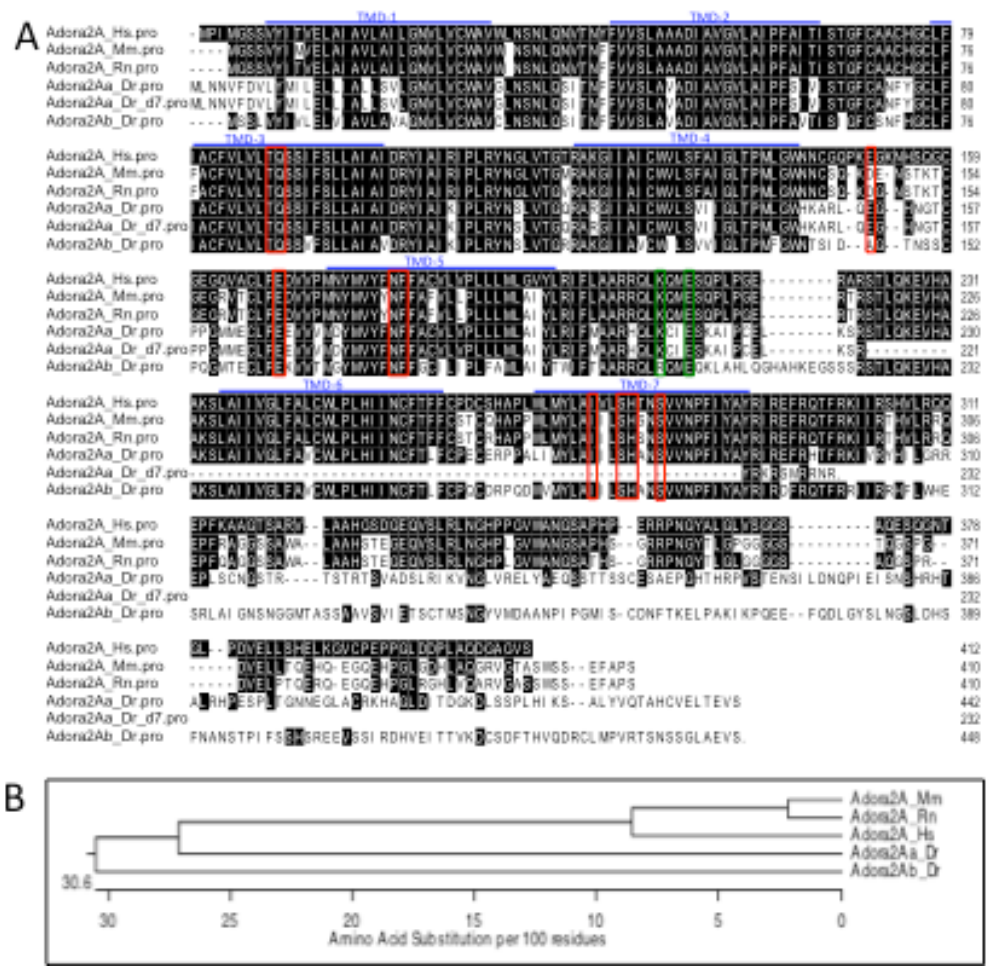

Figure 3.8: Adenosine A2A receptor and its homologs
Zebrafish (Dr) Adora2Aa, 2Ab (A) sequence alignments with the human (Hs), mouse (Mm) and rat (Rn) Adora1 respectively using clustal W analysis are shown in the figure. The alignment also includes the Adora2Aa d7 mutant sequence. Conserved residues are 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,

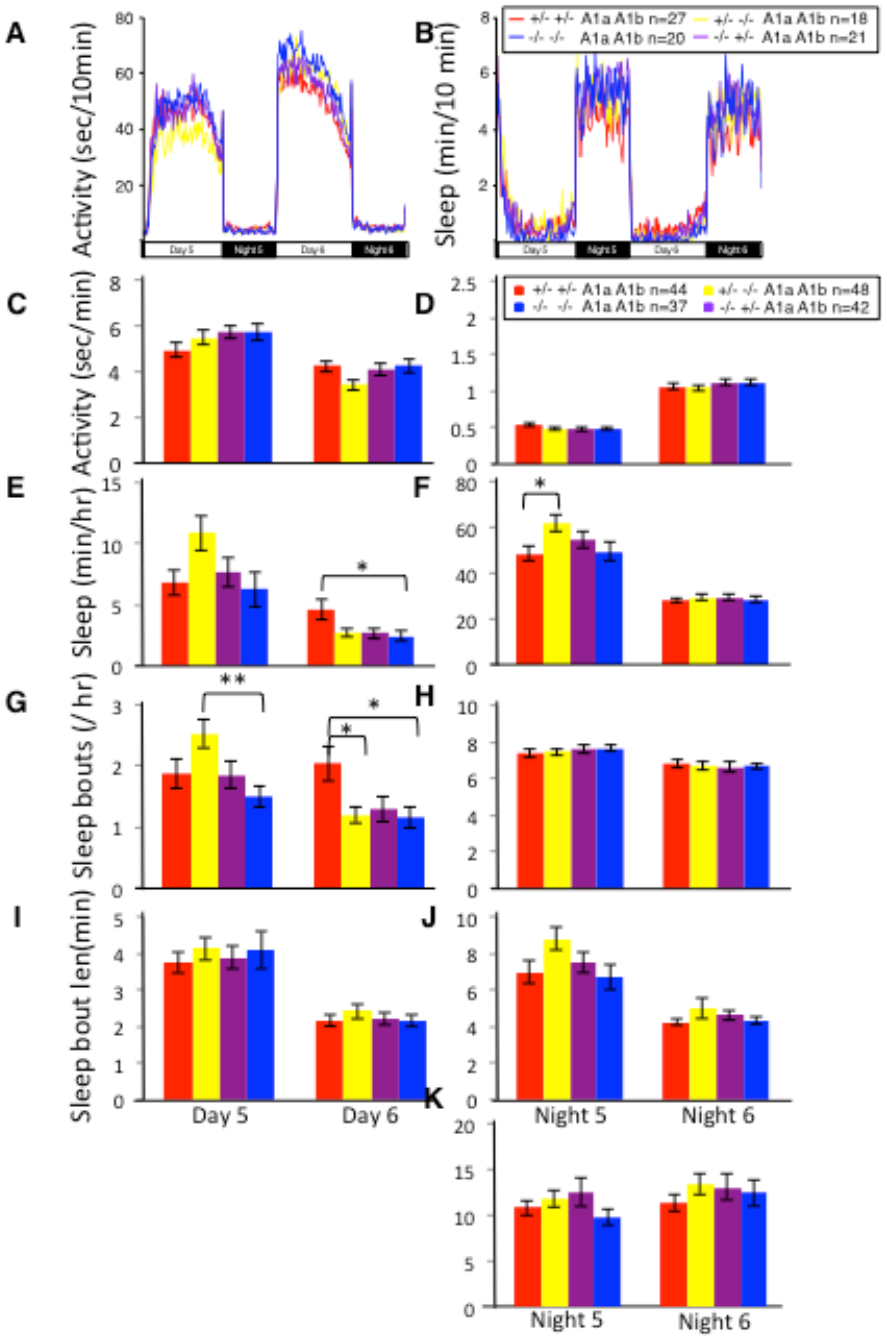

Figure 3.9: A1R mutants exhibit sleep/wake architecture similar to sibling controls A1R mutants A1a-/-;A1b-/-(blue) as well as A1a-/+;A1b-/- (yellow) and A1a-/-, A1b-/+ (purple) animals show comparable amounts of activity (A,C,D) , sleep (B,E,F), number of sleep bouts (G,H), length of sleep bouts (I,J) 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.

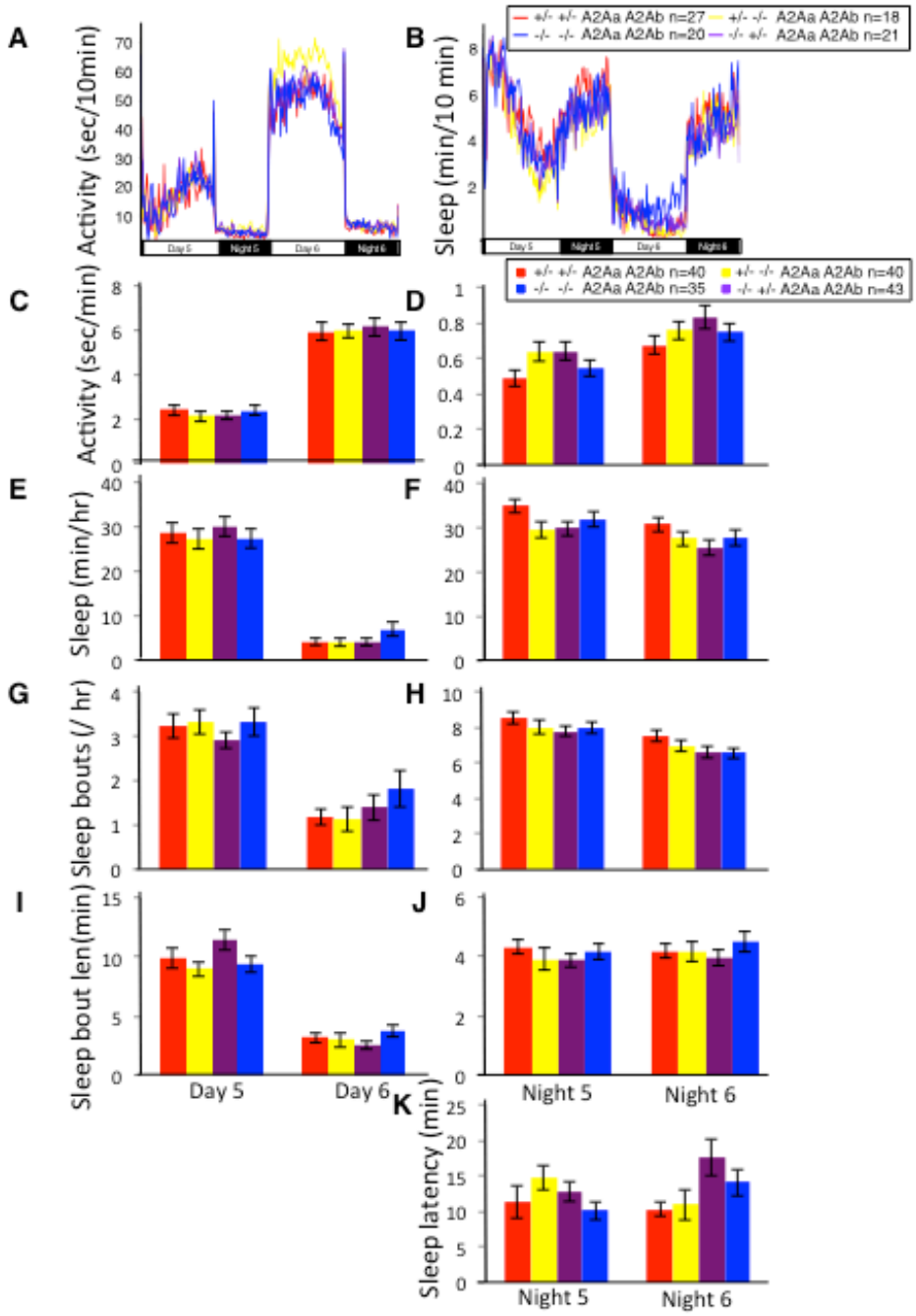

Figure 3.10: A2AR mutants exhibit sleep/wake architecture similar to sibling controls A2AR mutants A2Aa-/-;A2Ab-/-(blue) as well as A2Aa-/+;A2Ab-/- (yellow) and A2Aa-/-, A2Ab-/+ (purple) animals show comparable amounts of activity (A,C,D), sleep (B,E,F), number of sleep bouts (G,H), length of sleep bouts (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.

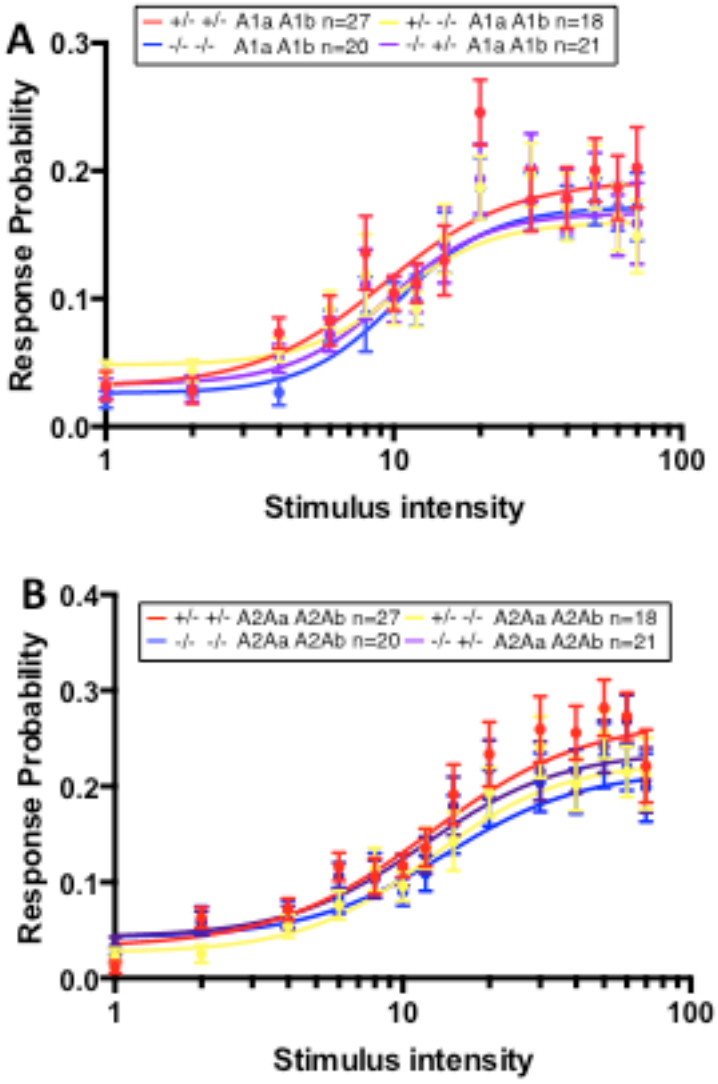

Figure 3.11: Adenosine receptor mutants exhibit similar sensory responsiveness. The response probability to a mechanoacoustic stimulus applied once per minute at night over a range of stimulus intensities is similar for all A1R mutant genotypes (log(half-maximal response probability)= 0.96±0.08, 1.013±0.07, 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.

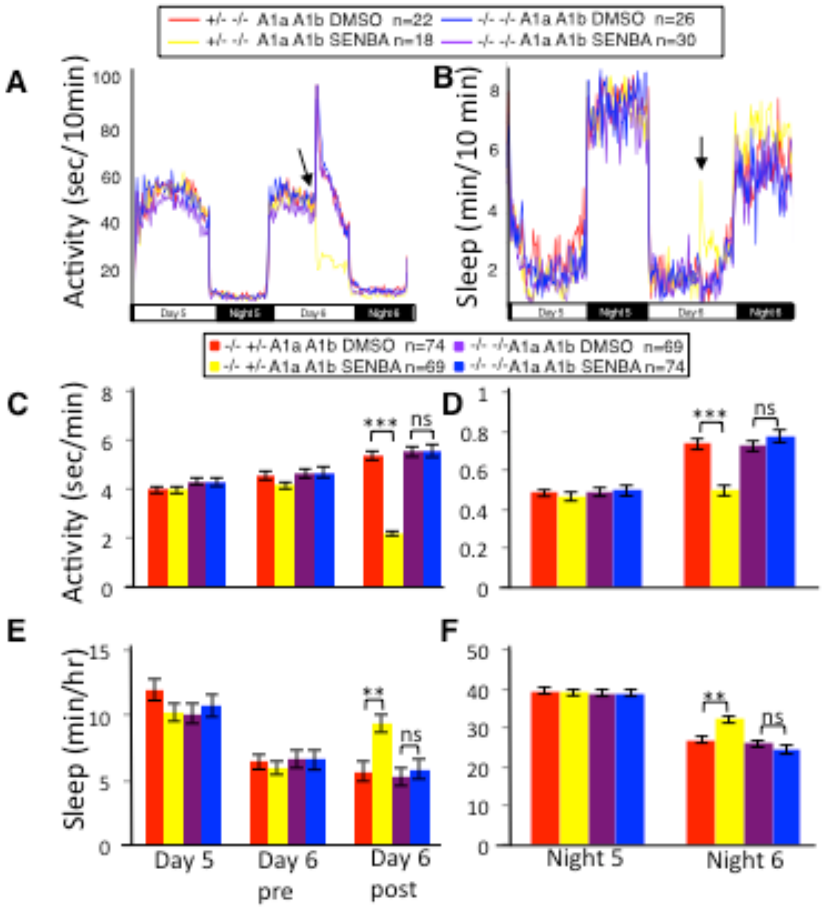

Figure 3.12: The zfA1aR is sufficient for the behavioral effect of theA1R agonist
Larvae were treated with DMSO vehicle or 20uM SENBA on day 6. After drug treatment, on both day and night 6, A1a-/+;A1b-/- animals display reduced activity and increased sleep as compared to vehicle treated animals (A-F, 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.

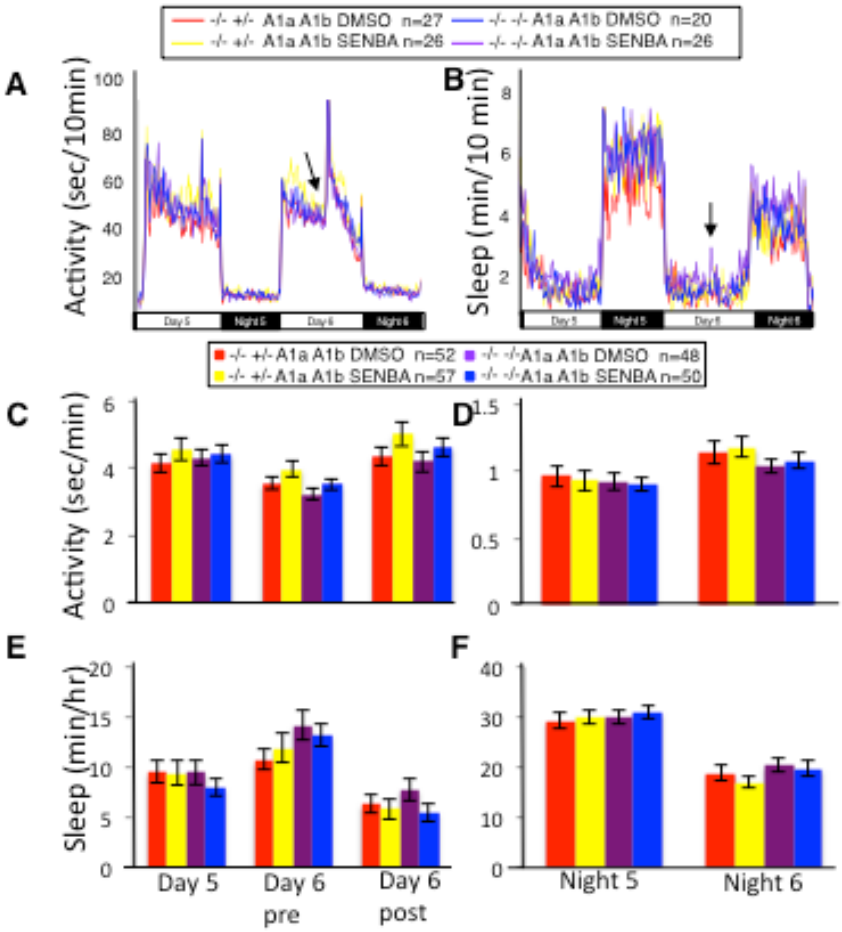

Figure 3.13: The zfA1aR is sufficient for the behavioral effect of theA1R agonist (A1a mutant background)

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.

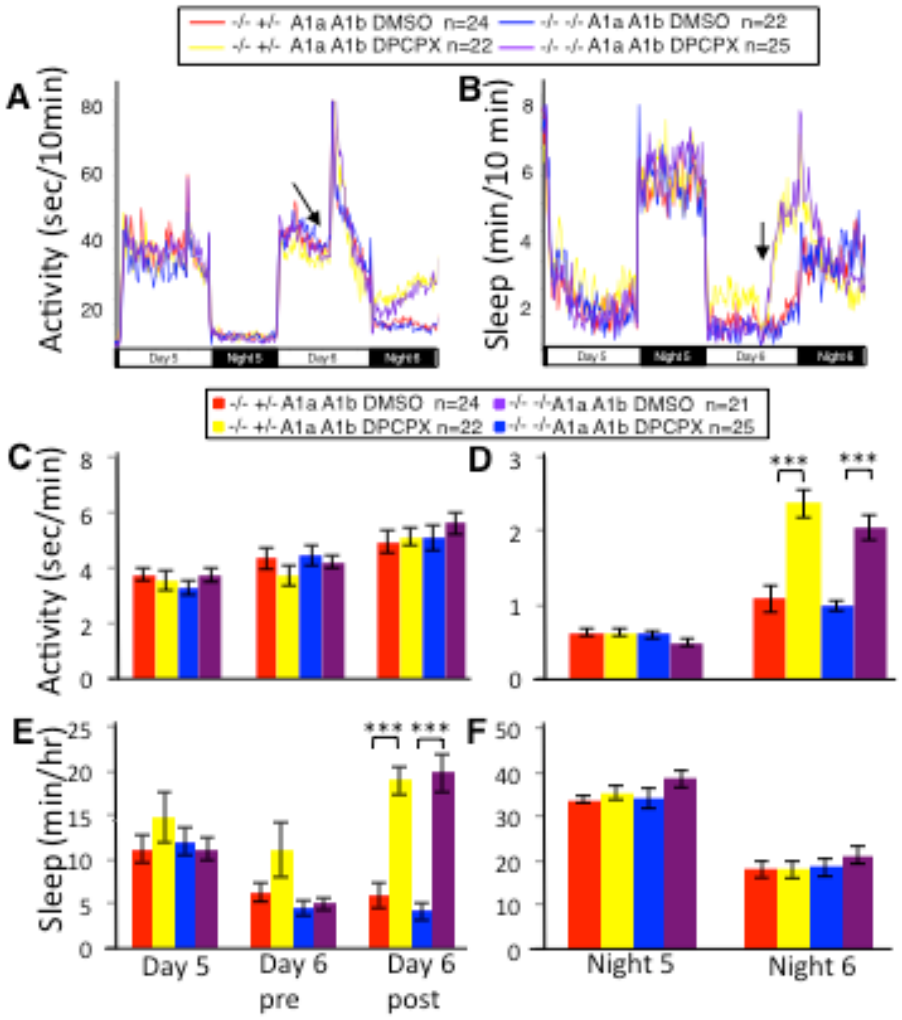

Figure 3.14: The zfA1aR and zfA1bR are not sufficient to mediate the effects of the A1R antagonist. Larvae were treated with DMSO vehicle or 20uM DPCPX on day 6. After drug treatment, on both day and night 6, A1a-/-;A1b+/- and A1a-/-;A1b-/- animals display increased sleep during the day and increased 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.

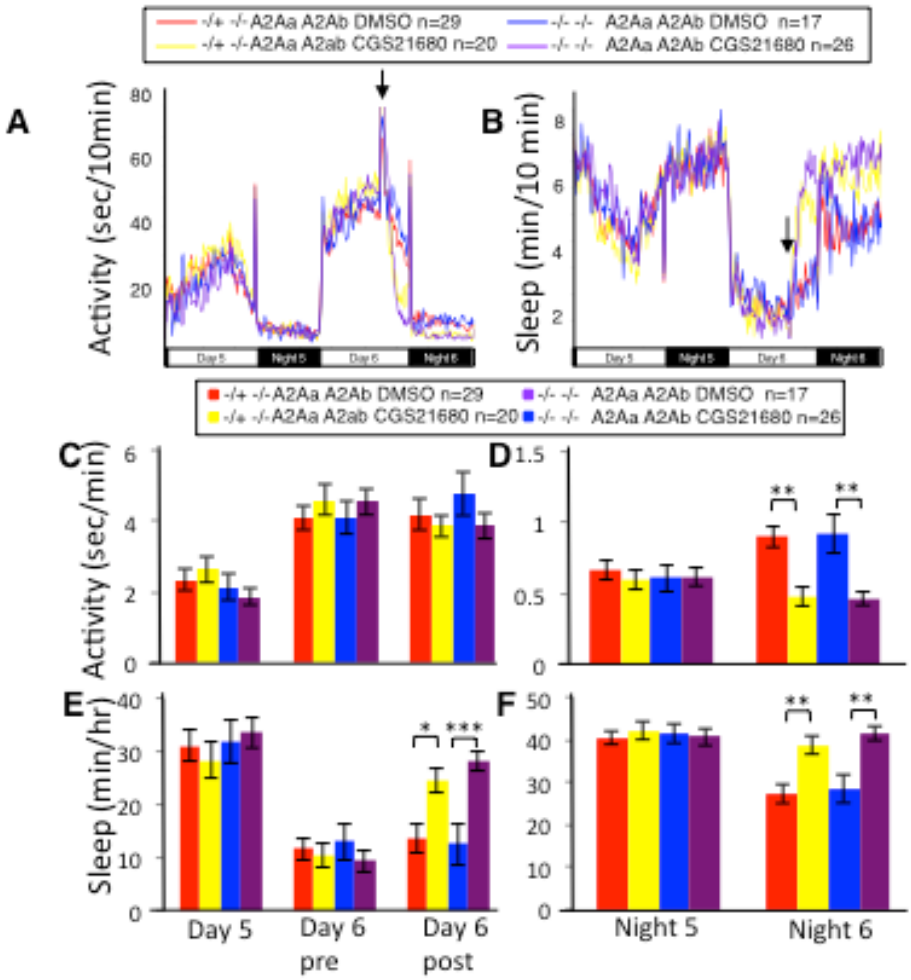

Figure 3.15: The zfA2AaR and zfA2AbR are not sufficient to mediate the effects of the A2AR agonist. Larvae were treated with DMSO vehicle or 150uM CGS21680 on day 6. After drug treatment, on day6 post treatment and night 6, A2Aa-/+;A2Ab-/- and A2Aa-/-;A2Ab-/- larvae sleep more during the day and night 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).

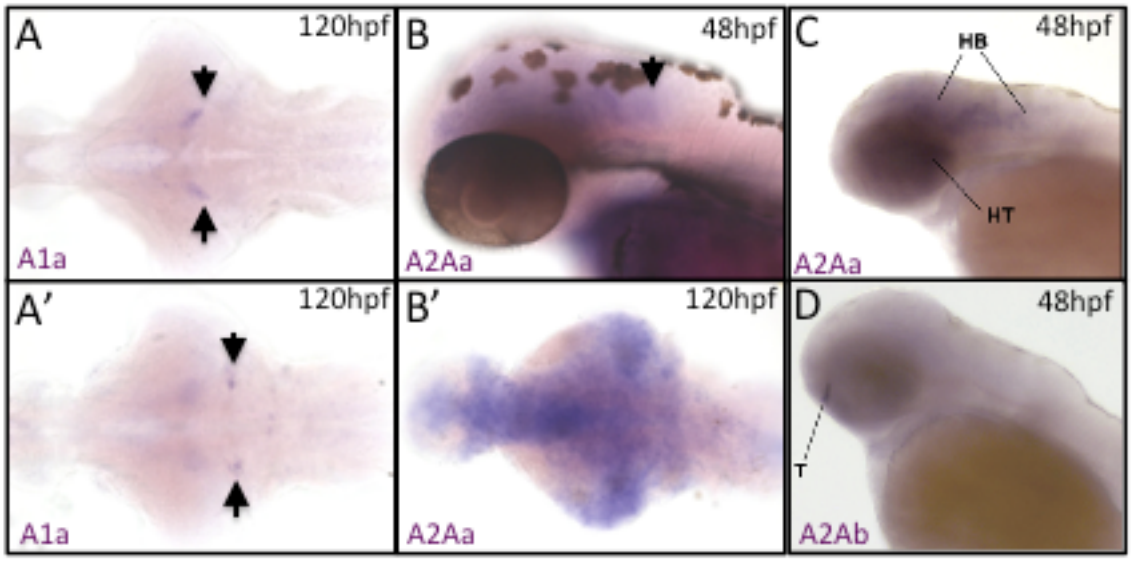

Figure 3.16: A1a, A2Aa and A2Ab expression in the zebrafish brain.
Insitu hybridization was performed with antisense probes against A1a and A2Aa. Faint A1a expression was observed in the rostral hindbrain and caudal hypothalamus area (A, A'). Faint hindbrain expression was detected in the hindbrain at 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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