EFFECTS OF ATOMOXETINE ON CIRCADIAN RHYTHMS AND LOCOMOTOR ACTIVITY

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Abstract

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Background: Attention deficit hyperactivity disorder (ADHD) is a common neurological disorder characterized by inattention, hyperactivity, and impulsive behavior. Many people with ADHD who are taking pharmacological treatments also report having sleep problems, and both children and adults tend to fall asleep later than neurotypical people. Since diagnosis and treatment of ADHD are on the rise, understanding how the disorder and common medications influence circadian rhythms is becoming more important. Zebrafish are a common animal model that have been shown to reliably represent features of many human disorders, including ADHD. **Hypothesis**: The specific hypotheses tested were: (H1) that a common medication used to treat ADHD, atomoxetine (ATX), would disrupt circadian rhythms in developing zebrafish; (H2) that ATX will increase locomotor activity overall; (H3) that ATX would influence light-evoked startle swimming; (H4) that treatment with 6 mg/L of ATX would have a greater effect on behavioral measurements than treatment with 3 mg/L. **Methods**: Zebrafish were raised from day 0-7 under a standard light/dark cycle to entrain circadian rhythms. Separate groups (N = 24 per group) were treated with an ATX solution with a concentration of 3 mg/L or 6 mg/L, or a control solution during entrainment. On day 7, locomotor activity was recorded for 5 minutes per hour for 24 hours in constant low illumination, followed by two brief (30 second) light-dimming evoked startle trials.

Results: The group treated with 6 mg/L showed altered circadian rhythms with higher overall activity levels across the entire recording period compared to the control group and the group treated with 3 mg/L. The group treated with 3 mg/L also showed significant differences when compared to the control group. All groups showed significant locomotor startle response to light dimming but startle response magnitude and duration were equivalent across groups. **Implications**: These results highlight the importance of understanding the effects of circadian disruption by ADHD medications. As a selective norepinephrine re-uptake inhibitor with no direct effects on brain dopamine levels, ATX is considered a non stimulant, but it still increased activity levels. This could be harmful to humans through sleep loss or other mechanisms. Future studies should examine the possible dose-dependent response to ATX in both adult and larval zebrafish. These results also justify using zebrafish to investigate the biological mechanisms of these drug effects on circadian cycles.

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Introduction

Neurological disorders are one of the most common disease threats in the world (Sejvar, 2017). Not only has the number of people with neurological disorders increased, but the number of deaths worldwide from neurological disorders increased by approximately 36% from 1980 to 2015. These findings highlight that neurological disorders are a threat to public health, and are an important subject for future research in terms of both treatment and prevention.

ADHD Background

According to Faraone et al. (2015), attention deficit hyperactivity disorder (ADHD) was first described in 1775 in Germany, but medicinal treatment was not discovered until 1937, and in the 1940's, the brain was discovered to be the cause of ADHD symptoms. In 1980, the third edition of the DSM created the first diagnostic criteria for ADHD. In 2013, the DSM-V categorized ADHD as a neurodevelopmental disorder, rather than a disruptive behavioral disorder. This led to an increase in the rates of diagnosis of ADHD.

As perhaps one of the most common neurological disorders in the world, ADHD is widely studied and characterized. The most prevalent symptoms of ADHD are hyperactivity, inattention, and impulsivity. These symptoms make up the current *Diagnostic and Statistical Manual of Mental Disorders-V* (DSM-V) diagnostic criteria for ADHD (American Psychological Association, 2013). Another common, although often overlooked, symptom is sleep problems and disorders (Hvolby, 2015), which will be discussed in more detail later in this paper.

ADHD tends to effect children more often and more severely than adults, though symptoms may persist into adulthood (Biederman, 2005). Children and adolescents with ADHD are at high risk of social dysfunction, poor academic achievement, and low self-esteem. Adults with ADHD show patterns that are typical with childhood ADHD, such as psychosocial disability and psychological dysfunction. People with ADHD are also known to have lower volume in several key brain structures, including the prefrontal cortex, corpus callosum, cerebellum, and the basal ganglia (Curatolo et al., 2010). In children specifically, there is a significant delay in cortical development, which is most notable in prefrontal areas. Additionally, children with ADHD are shown to have decreased cortical thickness (Shaw et al., 2012). Researchers are looking into possible risk factors for developing ADHD in order to combat these symptoms and deficits. Genetics are considered a strong contributor to ADHD, as well exposure to teratogens, such as nicotine or alcohol, which are chemicals or substances that can cause functional or physical abnormalities (Biederman, 2005). Pregnancy complications, such as toxemia and eclampsia, have also been considered risk factors for developing ADHD. Considering that approximately 5% of children worldwide are diagnosed with ADHD, and changing diagnostic criteria is making diagnosis in adults more prevalent (Faraone et al., 2015), research is focusing on the treatments of the disorder, in order to understand it further and therefore aid people with ADHD.

The most common treatments for ADHD in the United States are pharmacological (Faraone et al., 2015). Methylphenidate (MPH), a common stimulant medication for ADHD, has the primary action of being a dopamine reuptake inhibitor (Jaeschke et al.,

2021), binding to dopamine transporters, and blocking dopamine reabsorption by neurons. MPH also acts as a norepinephrine reuptake inhibitor, but with less potency than its effects on dopamine. Dopamine helps regulate processes like motor activity, learning, and memory, and deficits in dopamine transmission have been found to play a key role in ADHD symptoms (Stewart et al., 2015). MPH, and other stimulant medications, are generally the most effective pharmacological treatment for ADHD, but the type of treatment used also depends on the individual and the type of symptoms they present, as well as any comorbidities they may have (Faraone et al., 2015). For example, an individual with a cardiac disorder as well as ADHD would have to consult a cardiologist before taking any kind of stimulant medication. Stimulants are also unlikely to be used when the individual has bipolar personality disorder, depression, or anxiety, but there are other, newer options that have recently been shown to be successful in treating ADHD with these comorbidities.

Atomoxetine (ATX) is a medication that is commonly prescribed for children and adolescents with ADHD, and it was the first non-stimulant medication for ADHD approved by the U.S. Food and Drug Administration in 2002 (Fu et al., 2022). Unlike MPH, it is primarily a norepinephrine reuptake inhibitor. The process also passively increases levels of dopamine uptake in the prefrontal cortex, which helps alleviate the symptoms of ADHD. It is a relatively new drug, but research has shown that it is just as effective as MPH at regulating brain activity abnormalities and improving cognitive function in individuals with ADHD. Additionally, it is an effective treatment for individuals with co-morbid disorders, and in some cases, may also help alleviate

symptoms from disorders other than ADHD. Individuals with ADHD and anxiety have shown an improvement in their ADHD symptoms as well as improvements in their symptoms of anxiety with ATX. When deciding what medication to prescribe, clinicians conduct assessments of many aspects of the individual's life, including home life, severity of symptoms, and any known or possible co-morbidities (Faraone et al., 2015). Prescribing an individual a medication that is not effective, or that aggravates symptoms from a co-morbidity, could have serious side effects. This is why clinicians are interested in the differences between stimulants and non-stimulants, such as MPH and ATX. They could possibly cause different side-effects, including altered circadian rhythms and sleep quality.

Circadian Disruptions in ADHD

Another area of research into ADHD is looking into the relationship with circadian rhythms. In humans, circadian rhythms are 24-hour cycles that are maintained through a feedback loop composed of many genes and proteins, which are modulated by transmitters such as dopamine (Korshunov et al., 2017). Light exposure and other environmental cues are tied with each step in the feedback loop, which entrains the cycle.

Research has shown that there is a link between ADHD and sleep problems and disorders (Bijlenga et al., 2019; Bondopadhyay et al., 2022; Hvolby, 2015). Children with ADHD are more likely to feel tired during the day, and spend more time in shallow sleep, rather than deep sleep, than children without ADHD (Bijlenga et al., 2019). They also tend to have significantly higher rates of nightmares, and they score higher on the parent

report Child Sleep Habits Questionnaire (CSHQ) in terms of bedtime resistance, sleep anxiety and sleep onset delay (Bondopadhyay et al., 2022). In adolescents, symptoms of ADHD are associated with falling asleep later at night, less sleep, and insomnia (Bijlenga et al., 2019). Adults show similar patterns, with more short sleep than adults without ADHD. Adults also have higher rates of restless leg syndrome and insomnia (Bijlenga et al., 2019), as well as nocturnal awakening and activity (Hvolby, 2015). Studies that look at participants that take pharmacological treatments for ADHD show varying results (Hvolby, 2015). Stimulant medication has a calming effect by alleviating symptoms, such as hyperactivity, and promoting sleep quality. However, stimulants have also shown to increase sleep latency and to shorten the time spent asleep. Non-stimulant medication has been shown to increase somnolence during the day, but also shows decreased rates of insomnia when compared to stimulants. These paradoxical results from both stimulants and non-stimulants highlight the complicated relationships between sleep, medication, and ADHD.

The most common symptom among children, adolescents, and adults with ADHD is a delayed circadian rhythm. Individuals with ADHD have a delayed Process S, which refers to the desire or need to go to sleep, which then pushes back Process C, which is falling asleep and the circadian rhythms (Bijlenga et al., 2019). When individuals with ADHD then wake up at a normal time for work or school, this results in a sleep deficit. Other symptoms of ADHD may also be involved in pushing back Process C. Hyperactive behavior or constant running thoughts likely makes it hard to fall asleep, and impulsivity is associated with delaying sleep and lowered sleep efficiency. The full relationship

between sleep problems and ADHD is not understood, as they share many of the same neuroanatomical areas and behavioral symptoms, so a portion of research into ADHD involves trying to understand the role sleep problems have.

Animal Models of ADHD and Circadian Rhythms

This thesis aims to study how pharmacological treatments for ADHD affect circadian rhythms and hyperactivity using experiments on zebrafish, therefore it is important to discuss how animal models in general contribute to understanding ADHD and circadian rhythms. They are a widely used alternative to human participants when studying ADHD as they avoid common problems that occur in human participants, including lack of control over extraneous variables, strict ethical restrictions, and time constraints (Fontana et al., 2019). Researchers have commonly used Swiss mice (Costa et al., 2016) and fruit flies (*Drosophila melanogaster*) (Voet et al., 2016) to study ADHD. These animal models cannot be diagnosed with ADHD, however, they can display the phenotypes of the disorder that are similar or nearly identical to the phenotypes humans present. A phenotype is the observable behavior or symptom of a disorder, such as hyperactivity or inattention in regards to ADHD (Rommelse et al., 2008). Therefore, in order to have face and construct validity, an animal model of ADHD must present the basic phenotypes of ADHD - hyperactivity, impulsiveness, and sustained inattention (Fontanna et al., 2019). These phenotypes do not have to be present simultaneously in order for research to be conducted, which allows researchers to use animal models to study one aspect or symptom of ADHD.

There are over a thousand published studies using animal models to study ADHD. Animal models have been developed for the core behavioral symptoms of ADHD, including inattention (Bouchatta et al., 2018) and hyperactivity (Kim et al., 2017), as well as neurobiological mechanisms of ADHD, such as the role of the dopaminergic system (Huang et al., 2015), genetic risk factors for developing ADHD (Voet et al., 2016), environmental risk factors (Nellore & P., 2015), and drug treatments (Lange et al., 2012). The relevance of animal models is supported by findings that dopamine and norepinephrine pathways have similar effects on attention and hyperactivity in humans and other animals (Voet et al., 2016; Umehara et al., 2013), and that pharmacological treatments have similar effects on behavior and ADHD symptoms across species (Lange et al., 2012). Most importantly for this thesis, animal models of ADHD have also demonstrated similar circadian disruptions as seen in people, and similar circadian effects of ADHD medications.

The spontaneous hypertensive rat (SHR), which is a model of heart disease, can be used as an animal model of ADHD. They show several ADHD-like phenotypes, such as altered dopamine transporter, as well as altered circadian rhythms (Coogan et al., 2016). The SHR also exhibits poorer sleep quality, less time asleep, and waking up more frequently from quiet sleep when compared to a control group of Wistar-Kyoto rats (Kuo et al., 2004). Mice can also be used to model the relationship between circadian disruptions and ADHD. Researchers studied a mutant line of mice with altered circadian clock gene, *Per1*, and saw that they exhibited impulsive and hyperactive behavior, and they had impaired learning and memory (Huang et al., 2015). When exposed to nicotine

during development, several generations of mice have shown nocturnal hyperactivity and activity rhythms that resemble those seen in people with ADHD (Buck et al., 2019). When given MPH, these behaviors were brought down to baseline levels. Humans with ADHD that are treated with stimulants, such as MPH, often experience increased sleep latency, meaning the time they go to sleep is pushed back (Hvolby et al., 2015). Similarly, when male mice were treated with MPH in their drinking water, they experienced a delay in both activity and sleep, and the researchers also saw an increase in nocturnal activity (Antle et al., 2012). When mice were treated with ATX at a certain time of day, they showed delayed circadian rhythms (O'Keef et al., 2012).

Considering all this research, MPH and ATX may alleviate symptoms of ADHD such as hyperactivity, but they have paradoxical effects on circadian rhythms and sleep, which is shown through human research participants (Hvolby, 2015), and they affect the biology of circadian rhythms in animal models (Baird et al., 2013). A detailed discussion of animal models of circadian biology is beyond the scope of this thesis, but it is important to note that core features of circadian biology are deeply conserved across evolution (Bhadra et al., 2017), such as genetic and molecular feedback loops that maintain circadian rhythms within cells. Most of what is understood about human circadian biology comes from animal research, because these mechanisms are so conserved and similar. Sleep problems, or a lack of sleep, can lead to side effects that require medical treatment, hinder daily functioning, and exacerbate ADHD symptoms (Hvolby, 2015; Wajszilber et al., 2018). Further research is therefore needed on the relationship between ADHD medication and circadian rhythms.

Limitations of Animal Models

However, these animal models have limitations. In fruit flies, there is a specific gene that can cause ADHD phenotypes. These phenotypes can be mitigated by ADHD medication, however, this gene is not present in humans (Voet et al., 2016). Fruit flies also have a light-sensitive cryptochrome, unlike humans, that helps regulate daily activity. This could alter how they express certain ADHD phenotypes, such as hyperactivity. Costa et al. (2016) claim that Swiss mice develop some parts of the brain after birth, whereas in humans those same areas are developed during gestation. This means that studies that are conducted during gestation might overlook some possible effects. In addition, rodent studies are time consuming and expensive (Fontana et al., 2019).

Current Study

Zebrafish

This is why some researchers have started turning to zebrafish (*Danio rerio*). Zebrafish are easy to maintain, have a high rate of reproduction, at several hundred eggs per week, and have a high genetic similarity to humans (Santoriello & Zon, 2012). Zebrafish also fertilize eggs externally and have transparent embryonic and larval stages, which makes it easier to study their development (Stewart et al., 2015). The zebrafish genome has been entirely mapped out, and multiple genes have been identified and edited to develop mutant lines of zebrafish for genetic studies (Fontana et al., 2019). This also allows researchers to look into the genotype-phenotype relationship in zebrafish, and to then develop behavioral assays that can model neurological disorders in zebrafish

(Stewart et al., 2015). For example, the gene Latrophilin 3 (LPHN3) has been identified as an ADHD risk gene in humans, and the ortholog lphn3.1 has also been identified in zebrafish (Lange et al., 2012).

Zebrafish and humans share enough genetics and neurochemistry that they have become reliable models for human neurological disorders (Stewart et al., 2015). In zebrafish, the dopaminergic system, the system of dopamine pathways and transportation across the brain (Schweitzer et al., 2012), is highly studied and characterized in both larval and adult stages (Stewart et al., 2015). This makes it easy to conduct research, as well as make direct connections between the dopaminergic system in zebrafish and in humans.

Similar to humans, zebrafish with altered genes that are connected to developing ADHD show decreased activity at night than during the day, but they still showed greater activity levels overall than unaltered zebrafish (Lange et al., 2012). This was reminiscent of the hyperactivity shown by humans with ADHD, and the behavior became more similar to the unaltered zebrafish when exposed to ATX. Similar results were also present in zebrafish with an altered circadian clock gene (Huang et al., 2015). The zebrafish displayed hyperactive and impulsive-like behaviors, and had lower levels of dopamine compared to control zebrafish. These results suggest new avenues for research into zebrafish as a model for ADHD related biology and behavior.

Disadvantages

Zebrafish research still requires converging evidence, because they are still so different from humans. According to Stewart et al. (2015), zebrafish have more

neurotransmitter receptors than most species, including humans, due to an evolutionary genome duplication that occurred 450 million years ago. Additionally, this duplication resulted in zebrafish having two copies of some genes where humans only have one. Zebrafish also have different neural development and structure than mammals, which could pose challenges in developmental studies. Zebrafish do not have a neocortex, which has been implicated as an important part of dopamine projections and attention in mammals (Krauzlis et al., 2018). However, the overall structure and neurochemistry of attention is conserved between all vertebrates, as it developed before the evolutionary split. But conserved ADHD-related and circadian phenotypes discussed above overall support zebrafish as a model to study circadian aspects of ADHD.

The research presented here provides evidence to support zebrafish as a model for ADHD. Their dopaminergic pathways are highly conserved, and the zebrafish genome is so well studied that it is known that about 70% of human genes have at least one zebrafish ortholog (Fontana et al., 2019). Previous research has shown that zebrafish with altered circadian clock genes show increased locomotion, as both larvae and adults, during the daytime and the nighttime. Suzuki et al. (2020) looked at the effects of ATX exposure in adult zebrafish, and saw significant behavioral changes. They established that ATX can alter the behavior of adult zebrafish, but they did not include larva in their study. Therefore, this study will follow a similar procedure while studying larval zebrafish.

Hypotheses

My thesis proposal presented hypotheses to test two different ADHD medications, ATX and MPH, on circadian behavior in zebrafish. However, at the time I was not aware that a license was required to purchase MPH due to its classification as a stimulant drug. Therefore, testing with MPH was abandoned, and I instead compared behavior across three groups of zebrafish: high dose ATX, low dose ATX, and a no-treatment control. ATX treatment occurred from days 0-7 post fertilization, and behavior was tested on days 7 to 8 post fertilization. The specific hypotheses were tested:

Hypothesis 1: at both doses, ATX will disrupt circadian rhythms in developing zebrafish when compared to the control group.

Hypothesis 2: at both doses, ATX will increase locomotor activity levels in zebrafish overall when compared to the control group.

Hypothesis 3: ATX will influence light dimming-evoked startle responses.

Hypothesis 4: the higher dose of ATX (6 mg/L) will produce greater disruption in circadian rhythms and activity than the lower dose (3 mg/l).

Prior studies reviewed above have shown that ATX can disrupt circadian rhythms and increase activity (Suzuki et al., 2020), so Hypotheses 1 and 2 are supported as a theoretical replication and extension to a new model organism of those prior findings.

Methods

Zebrafish

All zebrafish handling and procedures were approved by the California Polytechnic University Humboldt Institutional Animal Care and Use Committee (IACUC). All zebrafish were housed according to previously established conditions (Matthews et al., 2002). Wild-type adult zebrafish (*Danio rerio*) were maintained in a 12-hour day/12-hour night cycle and in water kept at 28 °C. Adult male and female zebrafish were kept in separate tanks, and then placed together in a breeding tank that contained a mesh tray. At the beginning of the next day cycle, fertilized eggs were collected from under the mesh tray. The eggs were separated out into three groups: control, and 3mg/L ATX and 6mg/L ATX. Each group contained 24 zebrafish, as this was previously established as sufficient for statistical power analysis (Griffiths et al., 2012). No eggs were excluded from the study, as none had any noticeable malformations or fatalities before the study began. The sex of zebrafish embryos cannot be determined so sex was not a measured variable.

Design

Circadian rhythm was analyzed using a 3x2 mixed model ANOVA with drug condition (control, 3mg/L ATX, and 6mg/L ATX) as a between subjects factor and time (average activity during the 13 hours of subjective day and average activity during the 11 hours of subjective night) as a within subjects factor. Overall activity was analyzed using a one-way multifactorial ANOVA with 3 experimental conditions: control, 3mg/L ATX, and 6mg/L ATX. Startle responses were analyzed as a 3x3 mixed model ANOVA with

drug condition (control, 3mg/L ATX, and 6mg/L ATX) as a between subjects factor and startle stimulus phase (10sec period pre-stimulus, 10sec period during light dimming stimulus, and 10sec period post-stimulus) as a within subjects factor. Once the 7 days of ATX exposure were complete, the zebrafish were transferred into a 24 well plate containing standard fish water for the circadian activity recording. They were transferred at 11:30am, and recording began at 12:30pm. Spontaneous swimming behavior was video recorded for 5 minutes per hour for 28 hours in dim illumination, and the first four hours were considered habituation and removed from analysis. The image processing software ImageJ was used to calculate activity level of each larvae for each hour as the percentage of time spent in motion (for spontaneous activity, the percentage of video frames across the 5 minute epoch in which the larva's position changed from the previous frame) or as distance traveled (for startle responses, based on tracking the XY coordinate position of the center of the pixel density corresponding to the larva's body). Activity results from ImageJ were organized in Google sheets or Microsoft Excel before copying into SPSS or RStudio for statistical analyses.

Drug Exposure

ATX (atomoxetine hydrochloride; product number PHR1679) was obtained from Sigma-Aldrich, Saint Louis, MO. In previous research, behavioral tests have been run on zebrafish treated with varying levels of ATX. In this study, the zebrafish were continuously exposed to an ATX solution at 3 mg/L or 6 mg/L, or a control solution (standard swim water) in 60mm petri dishes (each containing about 20ml of liquid that was refreshed daily) for 7 days, starting at 0 days post-fertilization (dpf). This method is

based on previously conducted research (Lange et al., 2012; Levin et al., 2011; Suzuki et al., 2020). It should be noted that very few studies have been conducted with ATX exposure with larval zebrafish, so the amount used in this study is based on research involving adult zebrafish.

Measuring Circadian Rhythms

A circadian activity rhythm can be defined as the distribution of spontaneous activity across a 24 hour period, relative to the timing of the light-dark cycle during previous entrainment periods (Wolter and Svoboda, 2020). The expected circadian rhythm in control zebrafish would represent the way they were entrained, with more activity during the day and less at night. After the 7 day exposure period, the zebrafish from each group were recorded for one 24 hour cycle in constant dim illumination. Their activity levels were based on the amount of time spent in motion per hour, and subjective day and night time activity was the average per group, per hour, during what would have been day and night time in entrainment. Subjective day time and subjective night time activity were compared for each group using a factorial ANOVA.

Total Activity

The dependent measure of total activity level was defined as total percentage of time in movement across the 24 hour circadian activity test recording, and was compared across groups using a one-way ANOVA.

Startle Response

Two startle trials were run after the 24 hour spontaneous activity recording. Each lasted 30 seconds and consisted of 3 stages: a 10 second baseline recording, followed by

a 10 second recording during which ambient lights were extinguished, followed by a 10 second recording during which ambient lights were turned back on. Video captured one frame per second and image processing in ImageJ calculated the average distance travelled during each stage of the trial. Effects of the startle stimulus and drug treatment on swimming activity was analyzed using a 3x3 mixed model ANOVA with drug treatment as a between subjects factor and stimulus condition (baseline, startle, and recover) as a within subjects factor.

Results

Circadian Rhythms

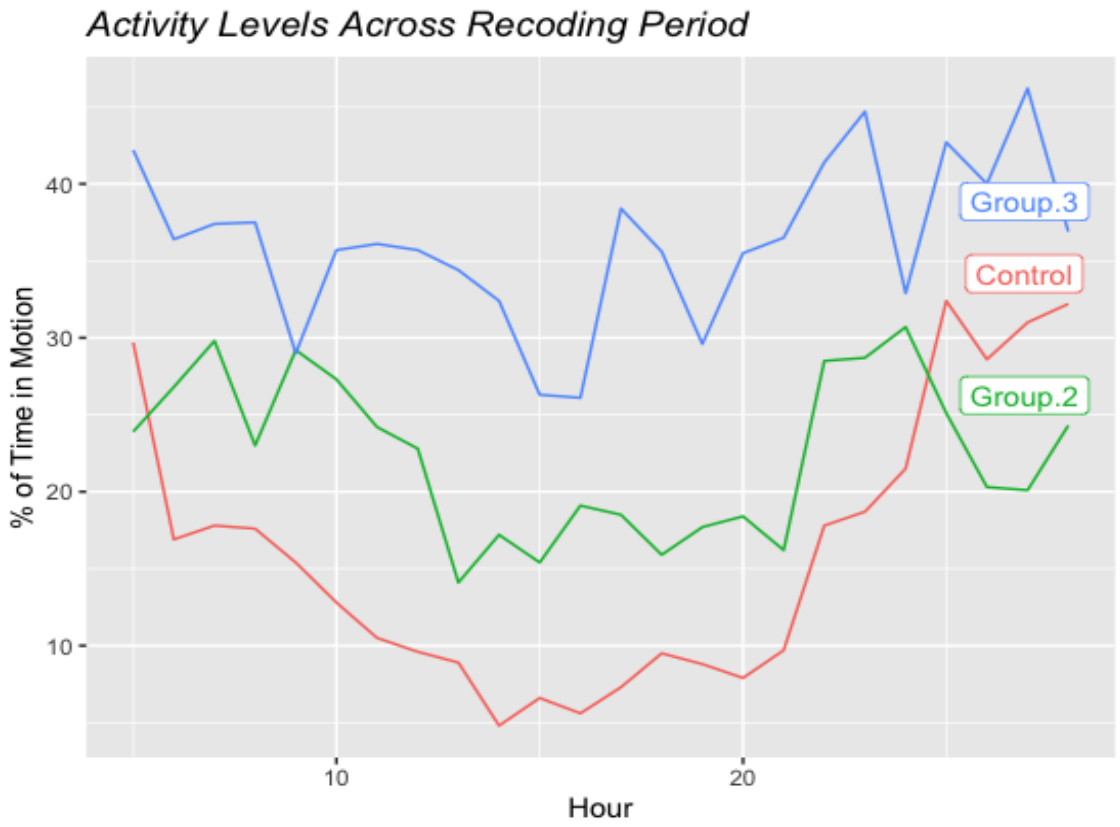
Subjective day time activity was compared to subjective night time activity across the groups. The results showed that there was a significant main effect for group ($F(1,66) = 174.31$, $p < .001$), and for time ($F(1,66) = 46.54$, $p < .001$), and a significant interaction effect ($F(1,66) = 8.68$, $p = .004$). Simple effects tests showed that across time, activity during the subjective day for the group exposed to 3 mg/L of ATX (M = 24.83) was higher than at night (M = 19.83; $F(1,64) = 8.99$, $p = .004$). For the group exposed to 6 mg/L of ATX, activity was also higher during the subjective day (M = 39.42) than night (M = 32.83; $F(1,64) = 15.59$, $p < .001$). Furthermore, simple effects tests between drug conditions showed that the higher dose of ATX had significantly higher activity during the day time than the lower dose of ATX ($F(2,64) = 63.57$, $p < .001$), as well as during the nighttime ($F(2,64) = 101.95$, $p < .001$).

Total Activity Level

Total activity level showed a significant difference in groups ($F(2,69) = 5.74$, $p = .005$) across the total recording period. A Dunnett's Test was run and showed that the groups exposed to 3 mg/L of ATX and 6 mg/L ATX had higher activity levels than the control group ($p = .003$; $p < .001$ respectively). A two-way factorial ANOVA was run comparing both experimental groups to each other, and there was a significant interaction found for group ($F(1,44) = 117.34$, $p < .001$), such that the group exposed to 6 mg/L of ATX showed higher ($M = 36.13$) activity than the group exposed to 3 mg/L ATX ($M = 22.33$).

A second factorial ANOVA was run to examine the effects of ATX on activity levels per hour across the total 24 hour recording period (see Figure 1). There was a significant main effect for group ($F(2,66) = 13.13$, $p < .001$), but not for time ($F(1,66) = 3.19$, $p = .08$). A Dunnett's test was run to compare the experimental groups to the control group, and significance was found for the group exposed to 6 mg/L of ATX ($p < .001$) , as well as for the group exposed to 3 mg/L of ATX ($p = .003$).

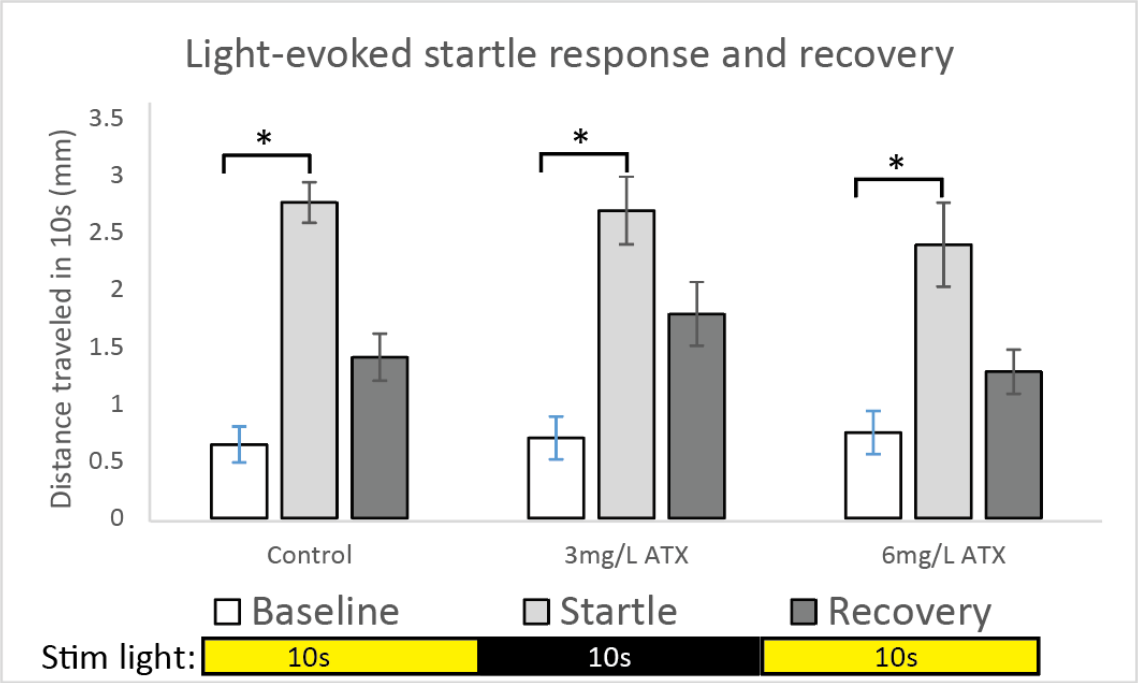
Figure 1



The total average activity levels for each group across the 24 hour analysis period. The activity levels for each well in the 24-well plates was averaged for each hour, for each group. Group 2 represents the group exposed to 3 mg/L of ATX, and Group 3 represents the group exposed to 6 mg/L of ATX.

Figure 2 shows startle responding to a 10 second lights-off stimulus relative to pre and post-stimulus behavior for each comparison group. A 3x3 mixed model ANOVA showed a significant effect of stimulus condition, with swimming activity higher during the 10 second startle stimulus than during the 10 second of pre-stimulus baseline or post-stimulus recover during which the lights were on ($F(2,132) = 56.70$, $p < .001$). However, there was no effect of treatment group and no group by condition interaction, suggesting that ATX did not influence light-evoked startle swimming.

Figure 2



Swimming distance across 10 seconds before (baseline), during (startle), and after (recovery) sudden dimming of ambient illumination, which is a reliable startle-evoking stimulus in zebrafish. All groups significantly increased swimming during the startle stimulus (* = $p < .001$), but there were no differences between groups.

Discussion

The purpose of this thesis was to study how a pharmacological treatment for ADHD, Atomoxetine (ATX), affects circadian rhythms and locomotor activity in zebrafish. There has been previous research involving zebrafish exposure to ATX (Parker et al., 2014; Suzuki et al., 2020), but mainly with adult zebrafish. My study replicated the core result that ATX disrupts circadian rhythms and increases motor activity in zebrafish.

Hypothesis 1

The results of this study are somewhat similar to previous research. Suzuki et al. (2020) found that eight days of exposure to 3 mg/L of ATX was enough to significantly alter the behavior of adult zebrafish in the novel tank test. In the current study, there were significant results found for the group exposed to 3 mg/L of ATX in circadian rhythms. While these studies used different measures of behavior and zebrafish at different stages of life, they both showed that 3 mg/L of ATX alters the behavior of zebrafish.

There were also significant results found for the group treated with 6 mg/L of ATX in circadian rhythms. They showed altered circadian rhythms compared to the control group. This study is possibly the first study to examine activity levels in zebrafish that have been exposed to 6 mg/L of ATX, so these results have important implications for future research. Previously, this amount of ATX has shown to reduce anticipatory responding during the five-choice serial reaction time task in adult zebrafish (5-CSRT)(Parker et al., 2014). This suggests that 6 mg/L of ATX reduces impulsivity, a common symptom of ADHD. However the results from the current study suggest that the

same amount of ATX delays circadian rhythms, which is also a common symptom of ADHD.

Hypothesis 2

There were significant results found for both groups treated with ATX, for overall activity level. These groups showed higher levels of activity compared to the control group, in total activity levels and for every hour recorded. They also showed a significant difference in activity between them. The group treated with 6 mg/L of ATX was more active than the group treated with 3 mg/L of ATX. Previously, research had not looked at the relationship between these levels of ATX exposure and activity levels, in either adult or larval zebrafish. This thesis could be a step in a new direction for ATX, and therefore ADHD, research on circadian rhythms. The results obtained here support the possibility of there being a dose-dependent reaction to ATX, and that the minimum dose required to alter behavior in larval zebrafish is less than 3 mg/L, as it is in adult zebrafish (Suzuki et al., 2020).

Hypothesis 3

We also analyzed light-evoked startle swimming, which can be interpreted as an anxiety-related behavior (Cheng et al., 2022), but found no change related to ATX that would indicate reduced anxiety. This is opposite to previous research that also analyzed anxiety-like behaviors, and found that zebrafish treated with ATX showed decreased anxiety (Suzuki et al., 2020). These opposing results could be due to differences in measurement and age of the zebrafish. Suzuki et al. (2020) measured anxiety-like behavior through the novel tank test. Adult zebrafish treated with ATX spent more time

in the top portion of a fish tank they were unfamiliar with, which suggests reduced anxiety. The light-evoked startle response is also a measure of anxiety-like behavior, however there was no difference in larva exposed to ATX. These two different measures of anxiety-like behavior could measure different levels of anxiety that are expressed at different ages. In the future, this should be considered when studying behaviors related to ATX.

Hypothesis 4

The results we obtained supported our hypothesis that 6 mg/L would show a greater disruption in circadian rhythms than 3 mg/L. This hypothesis was based on previous research that found that 3 mg/L was the minimum amount of ATX needed to alter the behavior of adult zebrafish, however, a greater amount of ATX was also correlated with altered behavior (Suzuki et al., 2020). The results from this study show that both 3 mg/L of ATX and 6 mg/L of ATX are sufficient to show significant differences in circadian rhythms and overall activity levels when compared to the control (see Figure 1). There was also a significant difference when comparing the experimental groups to each other across time, showing that 6 mg/L of ATX was associated with a greater disruption in activity and circadian rhythms than 3 mg/L of ATX. Like the results of total activity levels, this points towards a dose-dependent effect of ATX on larval zebrafish, and that larval zebrafish are just as susceptible to ATX as adults. Further research would have to be conducted analyzing the minimum amount of ATX that affect larval zebrafish behavior, and looking into if adult or larval zebrafish are better models for ADHD.

Limitations

There are several limitations in this thesis that could be addressed in future research. This research was also limited by the amount and type of ADHD medication that was able to be obtained. Enough ATX for a third experimental group would have been able to narrow down the minimum amount of ATX needed to alter behavior in larval zebrafish. This would allow for a greater understanding of the larval zebrafish response to ATX, similar to our understanding of the adult zebrafish response. Obtaining methylphenidate (MPH) would have also allowed us to compare a stimulant and a non-stimulant, and how the type of medication affects circadian rhythms. For future research, both MPH and ATX should be used, in order to broaden the field of research, and because MPH is still the most commonly prescribed medication for AHD (Jaeschke et al., 2021). More levels of ATX should also be examined, as this will help support or reject the possibility of a dose-dependent response to ATX, and find the minimum dose required to alter behavior if there is such a response.

Conclusions

The results from this study show important implications for future ADHD research. Most research utilizing zebrafish and ATX exposure uses adult zebrafish, so understanding how larval zebrafish respond to ATX would expand our understanding of both zebrafish development and the way ATX interacts with the brain. It would also further our understanding of the relationship between ADHD and circadian rhythms. In order to do this, more studies should be conducted looking at a larger window of circadian recording, to find the exact circadian rhythms of the experimental groups. More studies should also look at varying levels of entrainment, and examine if ATX exposure affects the rate of entrainment. These and other studies are necessary to increase our understanding of the relationship between ATX and circadian rhythms in larval zebrafish, which in turn increases our understanding of the relationship between ATX and circadian rhythms, and sleep as a whole, in humans.

People with ADHD often report having trouble sleeping, or with having a sleep disorder (Bijlenga et al., 2019; Bondopadhyay et al., 2022; Hvolby, 2015). However, it is not entirely clear just how reliable the rate of sleep problems or disorders in people with ADHD are. Parents of children with ADHD may over report sleep problems, due to heightened attention to behavioral issues, and adults with ADHD likely under report sleep problems (Yoon et al., 2012). Taking ATX is correlated with less frequent insomnia and quicker sleep-onset when compared to methylphenidate (MPH), but it is also correlated with increased occurrences of feeling tired throughout the day (Fu et al., 2022). This paradoxical relationship is further complicated by symptoms of ADHD being

exacerbated by a lack of sleep (Hvolby, 2015). When sleep is negatively affected by altered circadian rhythms, it can increase impulsivity, inattention, and other symptoms of ADHD. However, these symptoms can also negatively affect the amount or quality of sleep received. This relationship between ADHD, sleep, and ATX is complex and still not fully understood, however, it highlights the importance of finding ways to treat ADHD that do not negatively affect circadian rhythms.

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