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ABSTRACT

Increased levels of corticotropin releasing hormone (CRH) are associated with anxiety, as are decreased levels of serotonin (5-HT). Do 5-HT levels influence CRH production and anxiety? The ventral hippocampus is associated with anxiety behavior, contains cells expressing 5-HT receptors (including 5HT7R), and extends axons into CRH producing regions of the hypothalamus (Jalewa et al., 2014; Jiminez et al., 2018). Therefore, 5-HT responsive neurons may play a role in the anxiety response and CRH production. To analyze how 5-HT may influence anxiety, 5dpf *Danio rerio* were examined following application of a 5-HT7R agonist (AS-19) and antagonist (SB-258179). A distance assay was used to assess physical manifestations of anxiety response. It is hypothesized that separate application of 5-HT7R agonist and antagonist should result in a decrease and increase in the anxiety response, respectively. Furthermore, it is hypothesized CRH levels should increase or decrease in an inverse manner.

INTRODUCTION

Autism Spectrum Conditions (ASCs) are common developmental conditions that affect social behavior, gastrointestinal functioning, sleep wake cycles, sensory perception, and anxiety levels. ASCs are often comorbid with anxiety conditions, ADHD, and learning disabilities. To understand potential causes of this condition and its comorbidities, one must consider the underlying neurological factors. The cortisol production pathway, also known as the stress pathway, may have an effect on ASC symptoms as social anxiety is often the most noticeable symptom of individuals on the Autism spectrum. Stress is a response to an external cause while anxiety is an individual's specific reaction to stress; however, they are derived from the same neurological interactions. For the purpose of this paper, they will be referred to interchangeably. Therefore, the biological underpinnings of stress and anxiety are the primary interest of this study. While the stress hormone, cortisol, is widely understood, the neuromodulators and neurotransmitter communications within the production activation pathway are largely unknown. It is, however, known that stress and anxiety may be regulated by serotonin as the current treatments for anxiety conditions are Serotonin Reuptake Inhibitors (SSRIs) and Monoamine Oxidase Inhibitors (MAOIs) (Frazer & Hensler, 1999). These drugs prevent the metabolism of serotonin allowing more serotonin to be present in the synapse for uptake. Serotonin is often abbreviated as 5-hydroxytryptamine or 5-HT. For the purpose of this paper, it will be referred to as 5-HT. Our lab analyzes the extent to which 5-HT may be implicated in cortisol production and, thereby, stress behavior via cortisol production activator signal, corticotropin releasing hormone (CRH).

Part 1: Autism Spectrum Conditions

1.1 Characteristics of ASCs

ASCs is a more recent label applied to what had been Autism Spectrum Disorders; however, for the purpose of this paper, they will be referred to as Autism Spectrum Conditions. This new designation acknowledges the problematic language with which neurodevelopmental and mental conditions are referred. ASCs are clinically defined, according to the Diagnostic and Statistical Manual of Mental Health (5th ed.; American Psychiatric Association, 2013), by persistent deficits in social communication, interaction, and restricted or repetitive patterns of behavior, interests, or activities. Criteria necessary to meet diagnosis include symptom presence in early childhood, clinically significant impairment in daily functioning, and the symptoms must not be better explained by an intellectual disability (American Psychiatric Association, 2013). Furthermore, the severity level of each case can be assessed by categories, "requiring very substantial support," "requiring substantial support," and, "requiring support," which are defined by deficits in social communication and the presence of restrictive or

Figure 1. Severity levels for Autism Spectrum Conditions. Adapted from DSM-5 (2013).

Severity level	Social communication	Restricted, repetitive behaviors
Level 3 "Requiring very substantial support"	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/ repetitive behaviors markedly interfere with functioning in all spheres. Great distress/ difficulty changing focus or action.
Level 2 "Requiring substantial support"	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and who has markedly odd nonverbal communication.	Inflexibility of behavior, difficulty coping with change, or other restricted/ repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/ or difficulty changing focus or action.
Level 1 "Requiring support"	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful responses to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to-and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

repetitive behaviors (Table 1). There may also be some language impairment, intellectual impairment, accompanying behavioral disorders or genetic conditions which must be separately specified in diagnosis and may require additional intervention.

1.2 Prevalence and Causes

According to a 2019 study by the Center for Disease Control (CDC), it is estimated one in 59 children have Autism and it is approximately four times more likely for boys to be diagnosed than girls. The statistical likelihood of boys having ASC over girls is highly debated among professionals as it is argued that due to the framing of the condition in terms of the male presentation of symptoms, the female presentation may not be as obvious, or may even be different than that of their male counterparts (Milner et al., 2019). A 2013 study (Buescher, Ci-dav, Knapp, & Mandell) estimates that the lifetime cost pertaining to caring for an individual on the spectrum without a co-occurring intellectual disability is around $1.4 million and the lifetime cost for an individual with a co-occurring intellectual disability increases to $2.4 million. Furthermore, the annual cost of caring for individuals in the United States is $137 billion. (Buescher, Ci- dav, Knapp, & Mandell, 2013 as cited in Dawson & Bernier, 2013). These estimates were based on services and support received in addition to opportunity costs and productivity loss.

With such effects on daily function as displayed in ASCs, the high rates of prevalence, and the increased expenses in caring for an individual on the spectrum, research determining causes and most effective treatments is remarkably varied. Several theories, both environmental and genetic, have been proposed to produce ASC pathology, but many such theories have failed to gain significant backing by the science community. This supports the current belief that ASC

pathology is multifactorial. Some proposals of environmental factors that may induce symptoms include maternal immune activation during pregnancy and prenatal exposure to toxins (Varghese et al., 2018). One study suggests that five percent of genetic abnormalities corresponding with ASCs are caused by single gene mutations, around ten percent are duplications, inversions, extensive deletions, or translocations of chromosomes, otherwise known as copy number variants, and a majority of the genetic differences are polygenic risk factors from accumulation of mutations (Varghese et el., 2018). The seemingly multifactorial pathology of ASCs is thought to contribute to the spectrum of symptoms seen across individuals. Conversely, the expansive presentation of symptoms may contribute to the difficulty in pinpointing a singular cause.

1.3 Identification and Treatment

The symptoms in autism are specifically described as a continuum that is highly individual. This individuality makes identification and treatment difficult. However, to optimize outcome, it is important that the condition is identified and treated early. Identification of ASCs may occur as early as 18 months and is informed by developmental differences in behavior, such as failing to meet milestones. These milestones may include babbling and gesturing by 12 months, use of single words by 16 months, use of two-word phrases by 24 months (Filipek et al., 2000 as cited in Dawson & Bernier, 2013). Physical identifiers of ASC development are often present around 18-12 months, such as motor delays, development of unusual repetitive behaviors, atypical visual attention, easily shifted or disengaged attention (Landa & Garrett-Mayer, 2006; Iverson & Wozniak, 2007; Ozonoff et al., 2008; Zwaigenbaum et al., 2005 as cited in Dawson & Bernier, 2013). In addition to physical identifiers, social differences may occur, including deficits in social orienting, the ability to share a common focus on something with

another person, and imitation abilities (Mitchell et al., 2006; Nadig et al., 2007; Ozonoff et al., 2010; Pres- manes, Walden, Stone, & Yoder, 2007 as cited in Dawson & Bernier, 2013). Furthermore, loss of language or motor skills at any age is an indicator of an ASC, or some other developmental condition, and should be treated as early as possible.

In order to ameliorate symptoms in daily quality of living, early treatment is necessary. Therapy for ASCs may take the form of either a focused or comprehensive approach. A focused approach is an evidence-based therapy which works to ameliorate a particular symptom, while a comprehensive approach is one that is constructed to improve overall functioning and daily life. Commonly, some combination of the two approaches is utilized in treatment of ASCs to address specific problematic symptoms, such as excessive aggression, in addition to increasing long-term outcomes and daily functioning. Some current examples of popular therapy may include play therapy, Applied Behavioral Analysis (ABA) therapy, and Early Start Denver Model (EDSM) therapy. There is, however, backlash about certain treatments that are perceived as aiming to make people on the spectrum 'normal' rather than accepting the individual as neurodiverse and working to help the child with necessary life skills. Some other concerns about these therapies is they do not address longitudinal skills, like answering questions that change from year to year (e.g. "How old are you?") and how to self-regulate emotional changes. Despite these critiques, studies still support the use of the behavioral therapeutic models available now.

In addition to behavioral therapies, there are some pharmacotherapies which are often prescribed to individuals on the spectrum. Antipsychotics are often used for treatment of irritability in individuals on the spectrum and currently only risperidone and aripiprazole are approved by the FDA for use on individuals on the spectrum (Goel, Hong, Findling, & Jo, 2018). SSRIs are also commonly used; however, there is no current data to support beneficial impacts

on individuals on the spectrum who take them. However, because anxiety is such an evasive symptom, it is important to address pharmacologically how best to moderate its effects on an individual. Therefore, further research into treatment of ASCs with SSRIs is warranted.

Part 2: Cortisol and Stress

2.1 Functional Overview of Cortisol

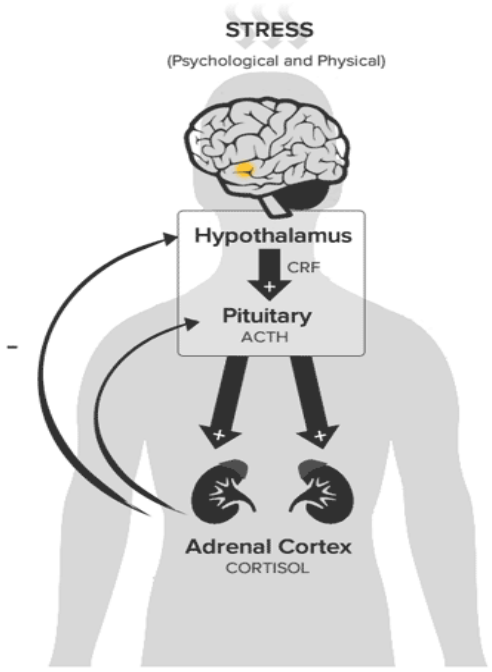
Cortisol is a glucocorticoid, which is a type of steroid that functions to modulate carbohydrate metabolism and is released during stressful situations (Brown, 1994). In addition to these functions, cortisol plays a role in controlling salt and water balance within the body, maintaining blood pressure, influencing memory formation, and aiding in fetal development (McEwen, 1999). Cortisol is also involved in some immunosuppressive and anti-inflammatory responses (Brown, 1994). Based on its involvement in many of the body's regulatory processes, it is evident that cortisol is a major contributor to healthy functioning and development; so, it's dysfunction can lead to serious health issues.

When there is too much cortisol over a prolonged period of time, sex drive may decrease and menstruation may become irregular, less frequent, or even stop occurring altogether. Too much cortisol may also lead to a condition called Cushing's syndrome, which is characterized by rapid weight gain in the upper body and face with slender arms and legs, flushed face, high blood pressure, easy bruising, muscle weakness, frequent feeling of thirst or need to urinate, and mood swings that may look like anxiety or depression (McEwen, 1999). Therefore, an increased cortisol can be problematic for healthy functioning. However, the reverse may also lead to dangerous health problems. Too little cortisol may be the byproduct of a problem in the pituitary or adrenal glands. Symptoms may include weight loss, fatigue, muscle weakness, dizziness,

mood changes, and darkening of regions of the skin (McEwen, 1999). The range of symptoms that occur from either too much or too little cortisol can significantly affect daily functioning and individual long-term health.

There is a long-standing association between varying cortisol levels and psychiatric conditions, like anxiety and depression. In fact, cortisol is often referred to as the anxiety hormone as it is specifically associated with the stress and fear response in higher vertebrates (McEwen, 1999). Evolutionarily, one may understand the need for cortisol as a way to physiologically prepare the body to respond to nearby danger stimuli; however, in modern times, such primal danger is no longer present, but the bodies have not yet evolved to deal with the reduction in danger stimuli. This may be why human conditions like anxiety and depression exist. It is important to understand the neurological basis of the cortisol pathway and analyze how it functions in animal models to better understand how to treat these conditions. There are several behavioral paradigms often used in laboratory settings to indicate levels of increased anxiety, stress, or fear as they all have similar biological derivatives. These paradigms include, but are not limited to, exploratory behavior, freezing, and startle responses (Schulkin, 1999). Exploratory behavior can be assessed by introducing an animal into a novel arena and assessing distance moved. Freezing behavior can be assessed at the same time as exploratory behavior in some cases and is marked by the inability to move. Startle responses can be measured by introducing some

Figure 2. Cortisol production stimulated by stressor. Modified from Alschuler (2016).

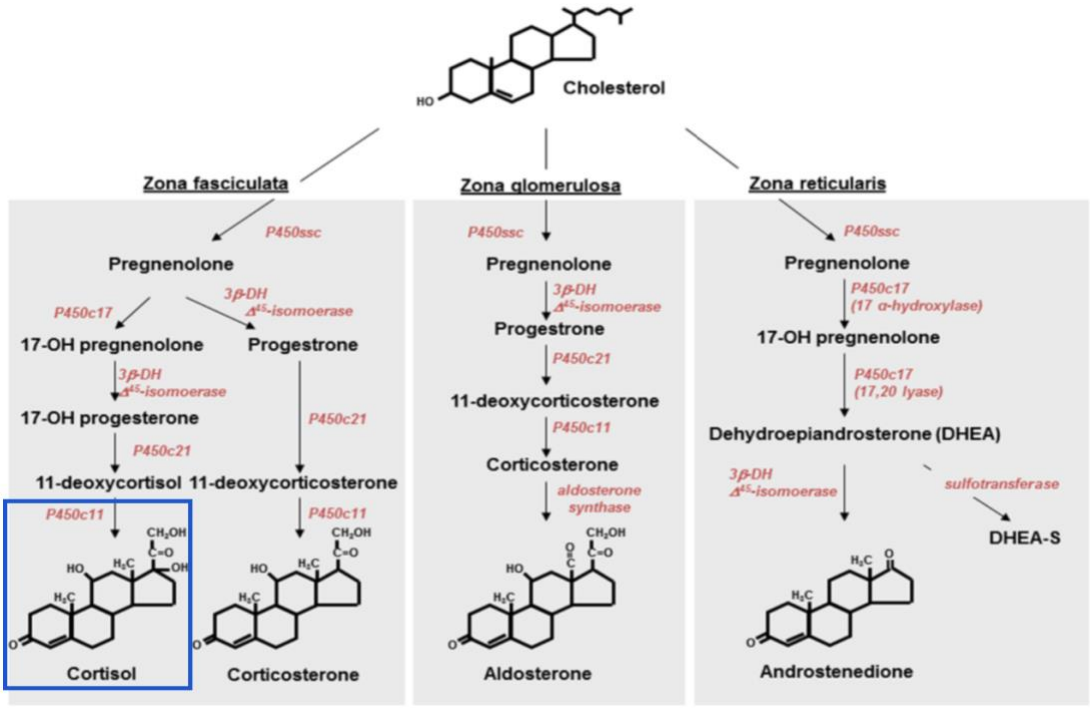


stimulus and analyzing the degree to which a known response is met (e.g. c-turn in zebrafish). With these paradigms, much about cortisol's behavioral effects can be learned and then applied to treatment of human conditions.

2.2 The Cortisol Production and Metabolism Pathway

Cortisol production begins when CRH in the paraventricular nucleus (PVN) of the hypothalamus is released which in turn signals the release of adrenocortical hormone (ACTH) in the pituitary gland (Figure 2). All subsequent chemical reactions to produce cortisol are catalyzed by enzymes. Cholesterol in the zona fasciculata layer of the adrenal cortex is signaled by ACTH to become chemically modified via enzyme into pregnenolone (Dhillo et al., 2002 as cited by Jones, 2017). Cholesterol, depending on in where it is found in the adrenal cortex and what enzymes are present, can make any steroid (McEwen, 1999). All steroids initially come

Figure 3. Biological pathway map of cholesterol forming steroid products. Cortisol production is highlighted in blue. Boldface and underlined words indicate layers of adrenal cortex. Adapted from Ortsater, Sjoholm, & Rafacho (2012).



from pregnenolone and then diverge into their respective pathways via specific enzymes (Figure 3). In the case of cortisol, pregnenolone is then catalyzed by an enzyme to become 17-OH pregnenolone, then 17-OH progesterone, followed by 11-deoxycortisol. Finally, cortisol is produced and released in the adrenal gland (Ortsater, Sjoholm, & Rafacho, 2012). After production, cortisol negatively affects the production of CRH and ACTH to stop further production of cortisol.

Steroids, including cortisol, when released, freely diffuse from the cell and through the phospholipid bilayer of another cell to a receptor within the cytoplasm of the receiving cell. The receptor/steroid complex then moves into the nucleus and functions as a transcription factor for particular genes (Figure 4; McEwen, 1999). This differs from other types of cell signaling which requires cell surface receptors, and the signal itself acts as a transcription factor rather than needing a receptor complex to enact change within gene expression. Following its production, cortisol is metabolized into 5-alpha-Tetrahydrocortisol and 5-beta-Tetrahydrocortisol (Jones, 2017). Often, cortisol and corticosterone are referred to synonymously; however, it is important to note that they are not chemically the same, but instead serve the same function within different organisms. Cortisol is found in humans, fish, and most other mammals while corticosterone is found in rats, mice, birds, and reptiles (McEwen, 1999).

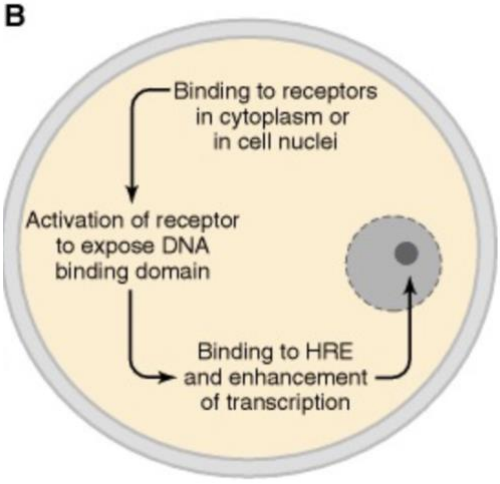


Figure 4. Steroid action in cell. Steroid binds intracellular receptor and is then transferred to nucleus as a receptor-hormone complex to activate or repress gene expression. Modified from McEwen (1999)

In research settings, it is important to be able to detect cortisol level; however, it is often difficult to do so in smaller organisms. In mice, rats, monkey's, and even humans it is common to detect cortisol by

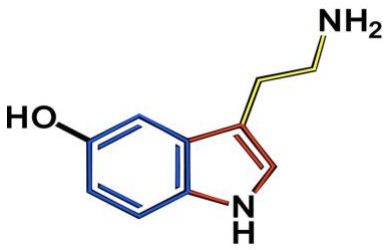
analyzing blood plasma or taking salivary samples (Lim, 2013). This procedure has also been done in fish, but in developmental research, the use of larvae or early staged embryos make collection of blood plasma difficult, as there is so little blood present to obtain data for analysis. For this reason, CRH can be used to detect presumed cortisol production. The metabolites of cortisol can also be analyzed to approximate cortisol production. (Olivereau & Olivereau, 1987)

Part 3: 5-HT and Cortisol Regulation

3.1 Functional Overview of 5-HT

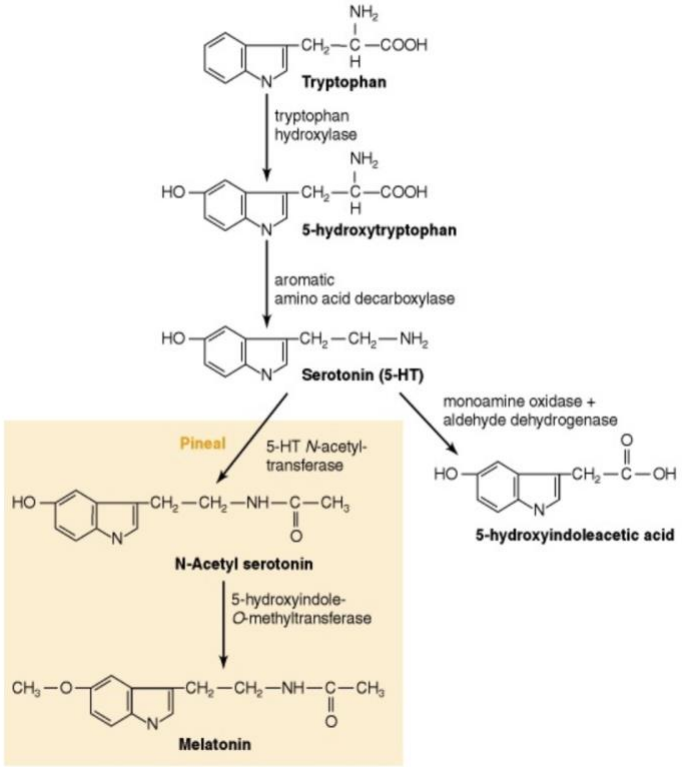
Serotonin, or 5-hydroxytryptamine (5-HT) is a neurotransmitter that is involved in a variety of functions including regulating the sleep/wake mechanism and eating behavior, gastrointestinal activity, social dysfunction, depression, and anxiety as well as some neuroendocrine regulatory activity (Frazer & Hensler, 1999). 5-HT is a monoamine, meaning it only has one amine group attached to its catechol ring and is considered a part of the catecholamine family of neurotransmitters (Figure 5). It is synthesized from L-tryptophan which is hydrolyzed to form hydroxytryptophan. Hydroxytryptophan is then decarboxylated to form 5-HT (Figure 6). After its production, 5-HT can either be catabolized to form 5-hydroxyindoleacetic acid or can be chemically modified to produce melatonin (Figure 6). 5-HT functions similarly to other amine transmitters and is released in a vesicle exocytotically into the synapse of a neuron and then interacts with its receptor on the postsynaptic membrane (Frazer & Hensler, 1999). It should be noted that there are 14 different known 5-HT receptor subtypes, all of which have g-protein coupled receptors with the exception

Figure 5. 5-HT chemical structure. Catechol (blue) attached to pentagonal hydrocarbon (red) with amine group (yellow). Image modified from Psychedelic Science Review.



of the 5-HT3 subtype which is a ligand-gated ion channel receptor. The different receptor subtypes are differentiated by their localization in the central nervous system (Frazer & Hensler, 1999). The known localization of each receptor subtype can help in finding novel drug targets for several conditions in which particular areas of the brain may be affected.

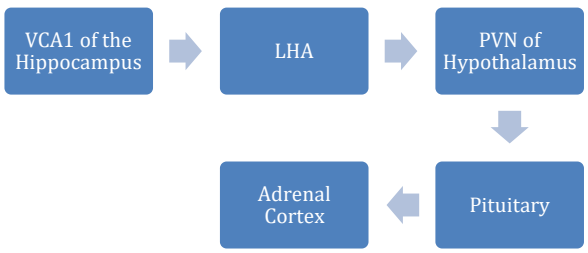
Figure 6. Serotonin synthesis and catabolism with chemical structure. Serotonin is also the precursor to melatonin in the pineal gland. Image adapted from Frazer & Hensler (1999).



3.2 A Proposed Role for 5-HT in an Anxiety Pathway

5-HT is commonly associated with anxiety and is often used as a drug target for treatment of associated conditions. As cortisol and 5-HT are both implicated in stress and anxiety, it is reasonable to suggest there may be some interaction between them. 5-HT axons have been shown to be found in several areas of the brain which connect with the signaling pathway that leads to cortisol production. The VCA1 of the hippocampus and the lateral hypothalamic area (LHA) both show presence of 5-HT and axons from the LHA extend to the hypothalamus where CRH is produced (Figure 7; Jalewa et al., 2014; Jimimez et al., 2018). It is then reasonable to assume that 5-HT may have some interaction with CRH production and may thus have some

Figure 7. 5-HT axons present in the anxiety pathway that interacts with cortisol production pathway.

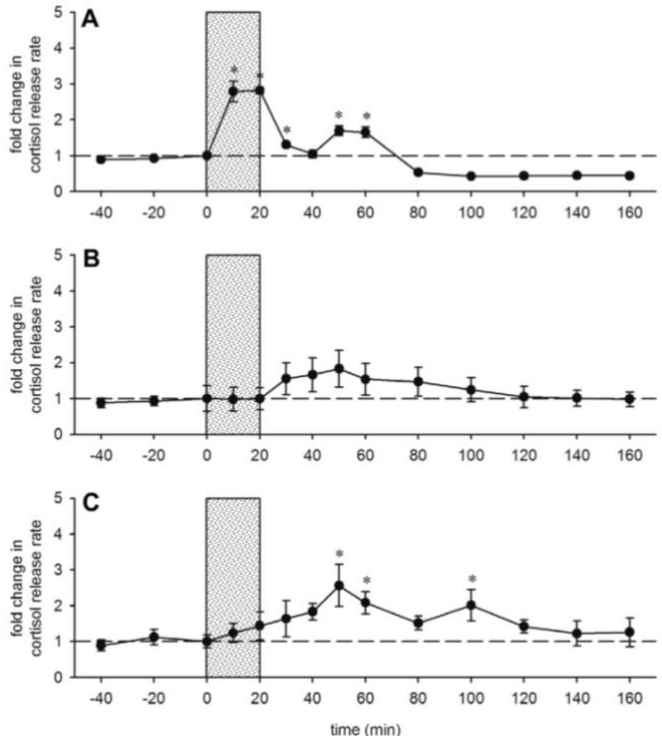


regulatory effect on cortisol production.

3.3 Targets for Manipulation

It is known that 5-HT stimulates the HPA axis; however, the exact sites of action are not well understood. In a 2013 study (Lim, Porteus, & Bernier), this lack of understanding was addressed via different intravenous agonist and antagonist treatments in goldfish. The agonist targeted 5-HT1A receptor subtype as well as the 5-HT7 receptor subtype. To address the agonist's ability to act on both receptor subtypes, separate antagonists for the 5-HT1A and 5-HT7 receptor subtypes were used. Following the treatment, ACTH and plasma cortisol levels were assessed. It was found that the 5-HT1A/7 receptor subtype mediated the largest effect. If the treatments had an effect on cortisol release rate, it would be expected that there would be an increase fold change of cortisol and the antagonist would cause a decrease in fold release rate, which is exactly what is shown. Based on these findings, we chose to further analyze the 5-HT7 subtype (Figure 8).

Figure 8. Data from Lim, Porteus, & Bernier (2013) study that indicates effect of 5-HT1A/7 (A) agonist and 5-HT1A antagonist (B) and 5-HT7 (C) antagonist on change in cortisol release.

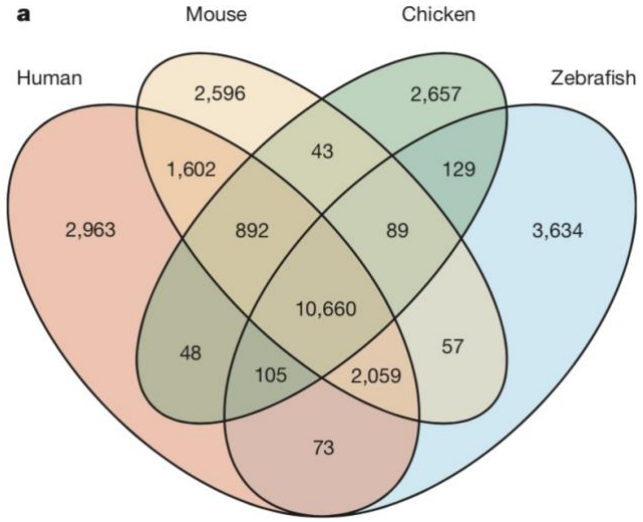


Part 4: Anxiety and Response in Teleosts

4.1 Teleosts as a Model System

In order to study and manipulate aspects of neural function that impact CRH/cortisol production, a model species, such as *Danio rerio,* must be used. The balance between simplicity of care and system complexity is well addressed by the zebrafish model. Zebrafish development is relatively fast, making it ideal for manipulation. By around five days post fertilization (dpf), *Danio rerio* larvae will have formed the entire body plan and have all basic organ structures in place; therefore, making them suitable for manipulation before adulthood is even reached (Kalueff et al., 2014). This early ability to manipulate gene expression, environmental conditions, or other conditions make zebrafish valuable to the scientist as they can be bred and experimented on quickly, allowing for high data production rates. They are also small in size, making them easy to house. *Danio rerio* maintenance is relatively simple in that they can be kept in tanks with other fish so socialization does not need to be managed by experimenters. Feeding and light cycles can be easily controlled. Zebrafish, however, also have relatively complex nervous systems that resemble human systems so they can be used as a model organism. The full genome of *Danio rerio* is known so they are highly genetically traceable, and approximately 70% of the human genome is orthologous to zebrafish genes (Figure 9, Howe et al., 2013). It is estimated that 82% of homologous zebrafish genes are orthologous to human genes that encode for disease-causing proteins (Shams et al., 2018). With such a high degree of similarity, it is

Figure 9. Number of orthologous genes shared between human (red), mouse (yellow), chicken (green), and zebrafish (blue). Adapted from Howe et al. (2013).

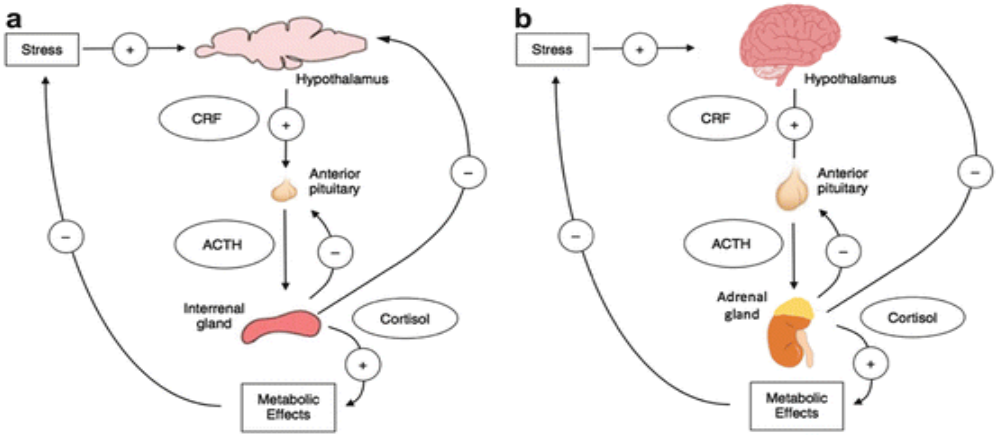


reasonable to assume that zebrafish genes may generate products that serve similar functions to those in humans. Furthermore, teleost species serve as an excellent model for human functioning, as the organization of brain structure is similar to that of humans (Kalueff et al., 2014). Zebrafish have all of the principle neuromediator systems which include synthesis and metabolism enzymes and neurotransmitter receptors (Kalueff et al., 2014). The genetic and physical similarities between zebrafish and humans further serves to support the continued use of *Danio rerio* in modeling of human conditions to determine pathology and novel pharmacotherapy.

4.2 Cortisol Production in Zebrafish

Current research suggests that *Danio rerio* cortisol response is correlated with stress behavior, as in humans (Kalueff et al., 2014). This is largely due to the evolutionary conservation of nervous system structures and endocrine tissues. Similar to humans, the teleost brain responds to stress by producing corticotropin releasing hormone (CRH) in the hypothalamus, specifically the hypothalamic nucleus preopticus (NPO). CRH then signals the production of adrenocortical hormone (ACTH) in the pituitary, which stimulates the production of cortisol from the head kidneys (Flik, Klaren, Van den Burg, Metz, & Huising, 2006). The head kidney is part of the interrenals in zebrafish, which functions similarly to the adrenals in

Figure 10. Comparison of zebrafish HPI axis (a) and human HPA axis (b). Adapted from Collier A.D., Kalueff A.V., Echevarria D.J., 2017.



humans. Likewise, the stress response is regulated by the hypothalamic-pituitary-interrenal (HPI) axis, instead of the hypothalamic-pituitary-adrenal (HPA) axis in higher vertebrates (Figure 10).

While cortisol production in *Danio rerio* mirrors that of human cortisol, there are several key differences. An important difference between teleost species and higher vertebrates is that CRH-containing cells in the NPO extend axons directly to the pituitary gland, whereas teleosts use localized release is more common. Therefore, higher vertebrates rely on receptors in the target area and fish rely on CRH-sending neurons to confer specificity (Flik et al., 2006). Another key difference in the cortisol production pathway is the method in which activation occurs. While teleosts may respond to perceived environmental threats in a way that mimics higher vertebrates, teleosts are also subject to alarm pheromones, which are pheromones secreted by their predators (Lim, 2013). Humans may not be as susceptible to, or even be able to detect, pheromones in this way and it should be considered that teleost species may have evolved to respond to predatory behavior more than humans when research is conducted on these subjects. Despite these differences, the cortisol production pathway in *Danio rerio* and other teleost species is still considered a useful model for human behavioral and neurodevelopmental conditions.

4.3 Zebrafish Models of Stress

Utilizing zebrafish for ASCs and anxiety disorders is extremely attractive because zebrafish behaviors associated with these conditions have been clearly defined. Recently, there has been a shift towards creating a comprehensive catalog of measurable behaviors and their relation to human behavior. Some assays associated with anxiety behavior are the c-turn assay, the novel tank assay, the light/dark assay, and measuring distance moved after a stimulus

(Kalueff et al., 2013). The c-turn assay consists of producing a stimulus and then analyzing the degree to which the organism turns in its escape maneuver. The higher the degree of the turn correlates with higher anxiety. Similarly, when a fish is placed in a novel tank, a sign of increased anxiety is the tendency to stay towards the bottom of the tank. Distance from the bottom can be measured to identify anxiety levels. The light/dark assay functions under a related premise, that a more anxious fish will tend to spend the majority of its time on the darker side of the tank rather than the lighter side and the amount of time spent in the dark versus the light can be measured as an indicator of stress. Finally, zebrafish tend to move away from a perceived threatening stimulus and the distance to which the fish moves away from the stimulus may indicate stress levels (Kalueff et al., 2013) There are several other stress and anxiety behaviors known that can be used to measure perceived stress which may be useful in further research.

Part 5: Hypothesis and Objectives

Stress and anxiety can cause a multitude of behavioral and mental problems that affect daily quality of life and may contribute to some symptoms in ASCs. It is known that 5-HT plays some role in anxiety and the production of cortisol, the stress hormone. It is with this in mind that the purpose of this study is to analyze how manipulation of the serotonergic system affects cortisol and, consequently, behavior in the model organism *Danio rerio*.

In order to address the implications of 5-HT in the production of cortisol and stress behavior, we used bath application of 5-HT7 receptor subtype agonist and antagonist on 5 dpf larvae after which behavior was analyzed through a series of behavioral assays, including c-turn and distance moved after stimulus, as well as immunoprecipitation to quantify the amount of CRH produced. It is hypothesized that with separate application of 5-HT7R agonist and

antagonist, there should be a decrease and increase in anxiety behavior respectively. Consequently, CRH levels should increase or decrease inversely compared to anxiety behavior. Thus, my research aims to provide a potential cause of anxiety behavior in ASCs by addressing 5-HT's role in the endocrine stress response in *Danio rerio* and addressing other possible functions in which 5-HT may be implicated.

METHODS

Fish Husbandry

AB wildtype-strain *Danio rerio* were obtained from ZIRC (Zebrafish International Resource Center, Eugene, OR, USA) and housed at 28.5°C with 10 hours dark, 14 hours light cycle. Zebrafish mating pairs were housed in separate 2.8 L tanks (Aquaneering, San Diego, CA, USA) and fed TetraMin tropical flakes (Blacksburg, VA, USA) every 12 hours and as needed for matings. The Pentair Shurflo water pump (Minneapolis, MN, USA) and Aquaneering water filter system (San Diego, CA, USA) were used to provide continuous flow of filtered, aged tap water. Mating were set 5-7 hours before the dark cycle was scheduled to begin. Males and females were placed in a mating tank separated by a clear divider. The divider was removed at the beginning of the next light cycle. Embryos were then collected and incubated in 1x E3 buffer at 28.5°C. Developmental stages were determined using staging criteria established by Kimmel et al. (1995).

Treatment Application

Agonist, AS-19 (Tocris, 1968), and antagonist, SB-258719 (Tocris 2726), were reconstituted in DMSO to 1 mM and stored at -20ºC until use. Five dpf larvae were treated with

62.5 uM agonist or antagonist for 22 hours at 28ºC (Lim, Porteus, & Bernier, 2013). DMSO alone was applied at the same concentration as that of the agonist and antagonist treatment to account for any potential effects of the solvent on embryo development. A control of 1x E3 buffer was also used.

Behavioral Assays

Following 22-hour incubation in treatment condition, single larvae were pipetted into individual wells of a 48-well plate and subjected to an auditory stimulus placed beneath the plate to analyze anxiety level. Specimen movement in response to auditory stimuli is used as a measure of anxiety level (Kalueff et al., 2013) The auditory stimulus was created using Garage Band (Apple). Response to the stimulus was filmed from above using an Edgertronic High Speed Camera. See Appendix I for further details about camera procedures. Larvae anxiety level was correlated with distance moved after audio stimulus. Distance moved was analyzed by using PHET (University of Colorado Boulder) and calculating the difference between coordinates. Averages of each larvae's movement were then calculated in Excel and plotted accordingly. Statistical comparison between treatment groups was also completed in Excel using the T.Test() function.

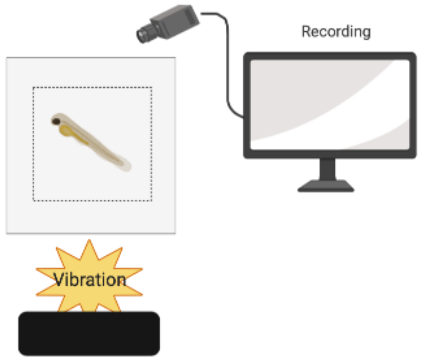


Figure 11. Camera and audio set up for behavioral analysis. Speaker was placed below the 48-well plate and response after stimulus was recorded.

Protein Analysis

Western Blots

A whole-body protein extract was produced from approximately 30 larvae homogenized in a chilled homogenizer in 1X RIPA buffer containing protease inhibitors. BCA assay was used to determine concentration of protein present within each sample. Protein extraction was then diluted and prepared for SDS-PAGE with sample buffer. Protein samples were run for approximately one hour at 106 volts. The gel was then transferred to PVDF membrane for immunoblot with anti-CRH rabbit polyclonal antibody (Boster Bio, A00629) and anti-GAPDH mouse monoclonal antibody(ABCAM, ab8245), as well as anti-rabbit IgG CF488 (Sigma SAB4600234) and anti-mouse IgG CF594 (Sigma SAB4600110) secondary fluorescently tagged antibodies. Membranes were imaged immediately following incubation using a UVP Imaging System. See Appendix II for further details on protocol.

Immunoprecipitations

Following a 22-hour incubation in treatment condition, approximately 30 larvae per condition were homogenized in a chilled homogenizer and whole-body protein was extracted in 1X RIPA buffer containing protease inhibitors. Protein extract was then immunoprecipitated with anti-CRH rabbit polyclonal antibody (Boster Bio, A00629) at a 1:100. Protein extract was also immunoprecipitated with anti-GAPDH mouse monoclonal antibody (ABCAM, ab8245) at a 1:1000 dilution as a control. Subsequently, Protein A sepharose was added for precipitation of the antibody and associated protein. Immunoprecipitants were separated gel electrophoresis using SDS-PAGE (BioRad, 4561083) for one hour at 106 volts. Following gel electrophoresis, protein was visualized using Silver Stain (Boster Bio, AR0171) following manufacturer protocol.

Images were obtained using a UVP gel imaging system. See Appendix III for more detailed protocol.

Densitometry

Images of silver stains gels were procured via a UVP gel imaging system and analyzed for differences in optical density between bands as a way to evaluate levels of protein present. Each band was analyzed using FIJI (Schindelin, Arganda-Carreras, & Frise, 2012). Background density was subtracted from each band to reflect accurate optical density. Visual comparisons were made but no statistical significance was calculated. See Appendix IV for more detailed protocol.

RESULTS

Effect of 5-HT Agonist and Antagonist on Behavior

Behavioral responses to auditory stimulus of 12 specimen per treatment condition for four separate trials were recorded and analyzed to assess the degree to which anxiety behavior was affected following treatment by agonist or antagonist. Responses were observed at two time points, 15 minutes post application and 22 hours post application. Individual larvae moved more in control treatment than agonist or antagonist treatments (Figure 12). Additionally, at 22 hours post application, there is an increase in movement for antagonist treated larvae compared to agonist treated larvae. However, no movement data were collected for DMSO because they died upon application.

To assess statistical significance of observed differences in distance moved, averages of each treatment condition were taken and analyzed by two-tailed pairwise t-test (Figure 13). T-test results indicated that there was statistical significance between the control treatment and the agonist ($p = 2.9 \times E\text{-}5$), the control treatment and the antagonist ($p = 0.00011$), and between the agonist and antagonist ($p = 6.8 \times E\text{-}5$). While statistical significance was indicated between untreated control and other treatments, the DMSO control, resulted in death and, therefore, zero movement. Thus, only the comparison between agonist and antagonist can be addressed as significant because they were treated with the same concentration of DMSO. The significance observed between agonist and antagonist is consistent with our hypothesis and further

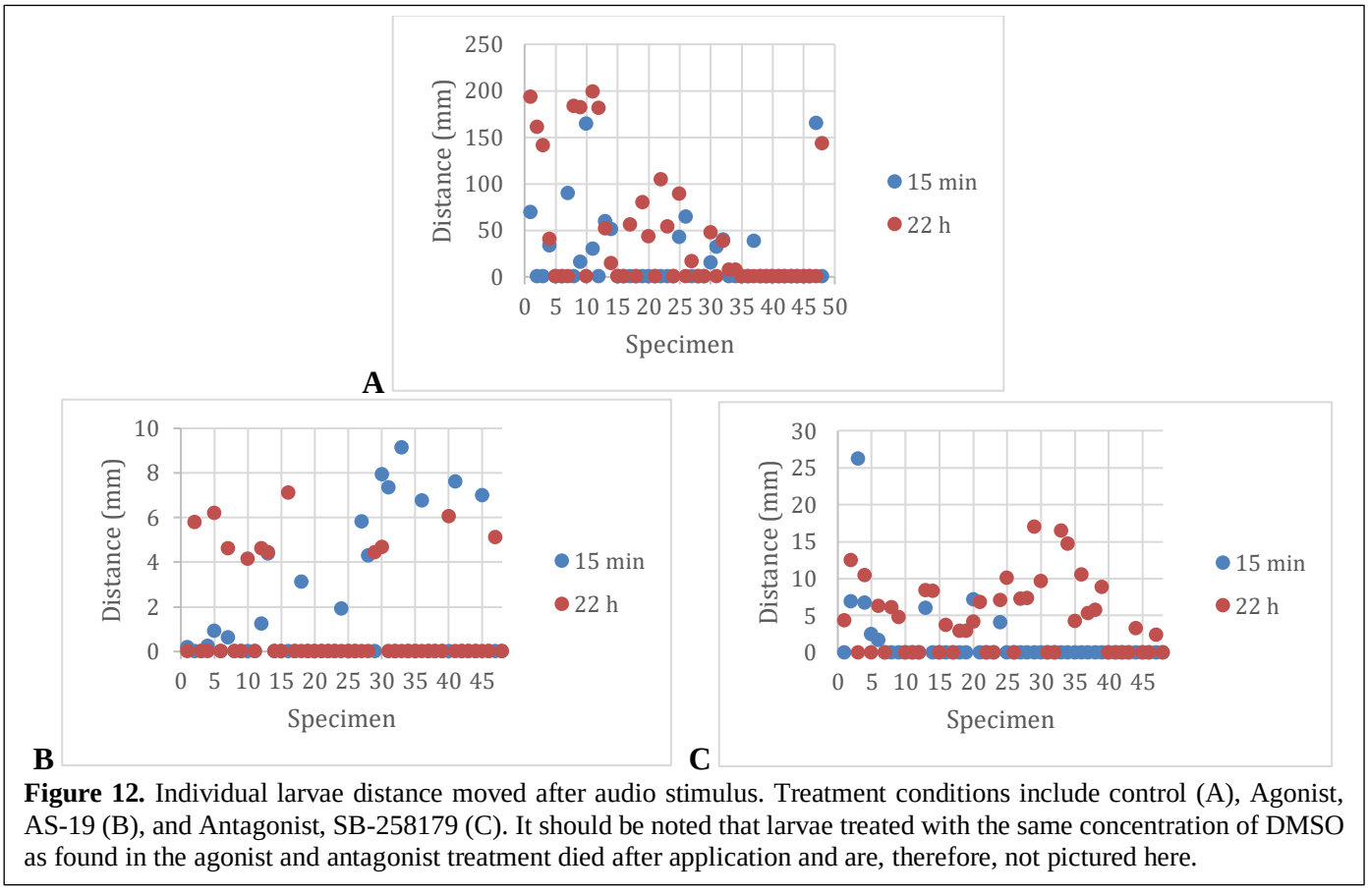


Figure 12. Individual larvae distance moved after audio stimulus. Treatment conditions include control (A), Agonist, AS-19 (B), and Antagonist, SB-258179 (C). It should be noted that larvae treated with the same concentration of DMSO as found in the agonist and antagonist treatment died after application and are, therefore, not pictured here.

replications should be done to address the effect of the solvent used for reconstitution on behavior in addition to the effect of agonist and antagonist on behavior.

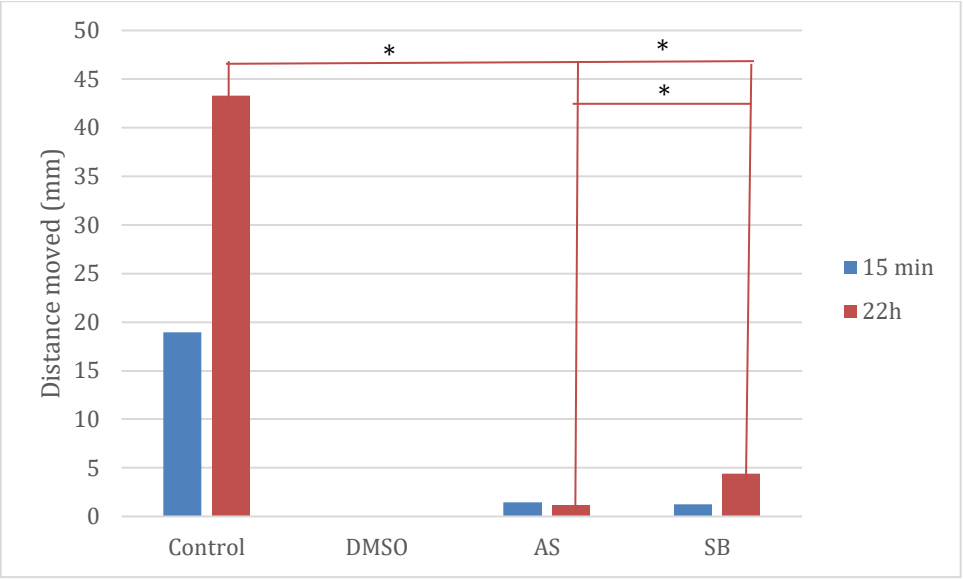


Figure 13. Average distance moved after audio stimulus per treatment. Time at data collection were 15 minutes (blue) and 22 hours (red) post application. Statistical significance is indicated by * for a p-values < 0.001.

Protein Analysis of 5 dpf Larvae

Western Blots

Initial attempts to detect changes in CRH levels used fluorescent Western blot analysis with wild type larvae. A number of variables were altered in numerous experiments, but there was no condition in which CRH protein was detected nor was the constitutively expressed protein GAPDH. To ensure the imager could detect the fluorophores used, dot blot tests using secondary antibodies were conducted. Fluorescence was observed in these tests for blots rinsed in TBST before addition of antibody (Figure 14).

Following evidence the imager could detect the fluorophores being used, we tested several different loading amounts of protein to assess whether there was not enough protein present. None of the amounts use resulted in detection of protein. It was then considered that

freezing the extract affected protein antigenicity. We repeated treatment assays in order to compare fluorescent western blot analysis using fresh and frozen samples. Once again, no protein was detected.

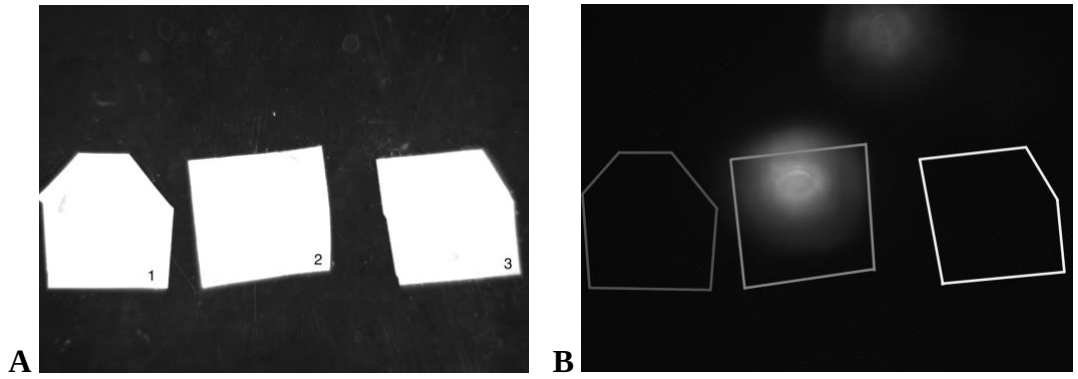


Figure 14. Dot blot test for Western blot. Whitefield image of dot blot (A) with methanol rinse before antibody addition (1), TBST rinse before antibody addition (2), and dry then TBST rinse before antibody addition (3). Fluorescent image of dot blot (B). Fluorescence is observed only with the A2 method.

Immunoprecipitations

Immunoprecipitation was used as a second method to detect changes in CRH levels. Previously prepared protein extracts (which were stored at -80ºC) from specimens used in behavioral assays were incubated with anti-CRH and anti-GAPDH antibodies followed by immunoprecipitation with Protein A sepharose. Immunoprecipitants were separated by SDS-PAGE and silver stain was done to visualize proteins. Although protocols indicated that protein extract could be frozen at -80ºC or used immediately following extraction, initial results detected little protein (data not shown). To make a direct comparison between frozen protein extract and fresh protein extract, two immunoprecipitations were run, one with frozen protein extracts and one with fresh protein extracts. Immunoprecipitation conditions were run at the same time, following the same protocols, and using the same reagents. It was found that the protein extracts

lost antigenicity after freezing (Figure 15). Therefore, following immunoprecipitations were begun with fresh extracts.

Treatments were repeated to generate fresh protein extracts to begin subsequent immunoprecipitations. In an effort to maximize the number of immunoprecipitations that could

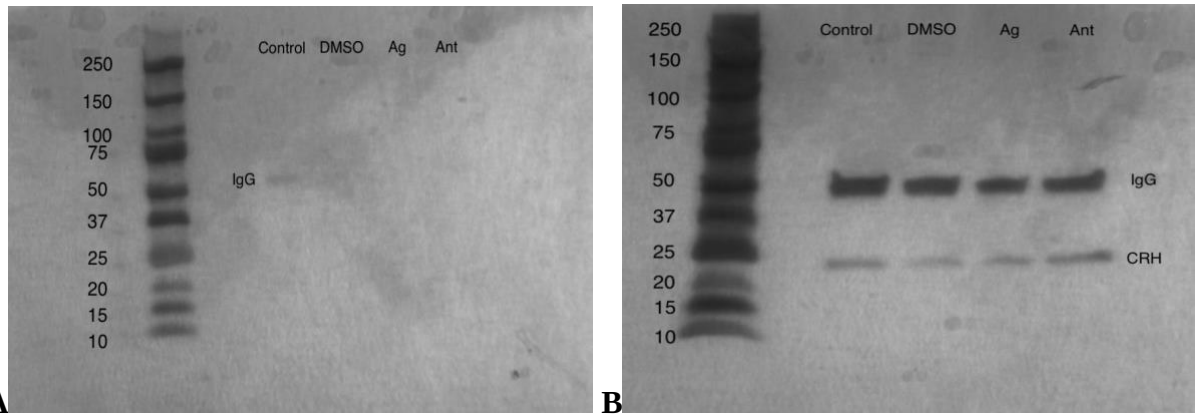


Figure 15. Immunoprecipitation with frozen protein extract (A) versus immunoprecipitation with fresh protein extract (B). Faint band in A indicate IgG at approximately 50 kDa, while clearer bands in B indicate IgG at approximate 50 kDa and CRH at approximately 23 kDa.

be analyzed, samples, after immunoprecipitation but before separation by SDS-PAGE and silver stain, were placed in sample buffer and stored at -80ºC. However, silver stain of gels with samples treated in this way detected no proteins other than the protein markers. Treatments were one again repeated and generation of protein extracts, immunoprecipitation, SDS-PAGE, and silver stain were done in succession without storing samples at any step. To maximize use of the protein extracts generated, both anti-GAPDH and anti-CRH antibodies were added simultaneously to these extracts. Unfortunately, a loading error with marker proteins affected gel quality. After silver stain of this gel, IgG (about 50 kD) and GAPDH (about 37 kD) were clearly visualized, and, to a lesser degree, it appears some CRH (about 23 kD) was detected (Figure 16). Plans were to repeat the procedure, but, due to time constraints, no further repetitions of treatments and immunoprecipitations were possible to try and resolve inconsistent results.

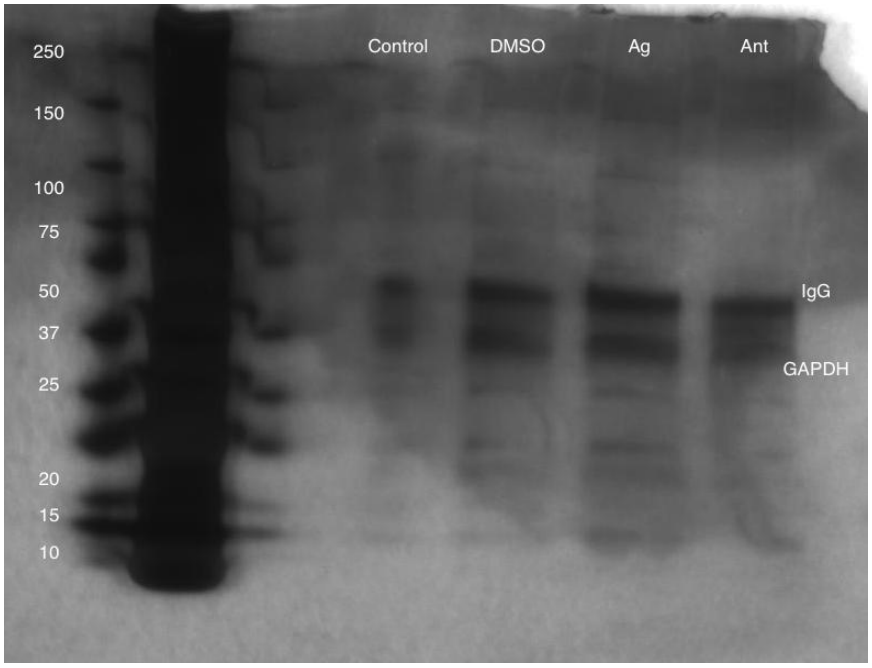


Figure 16. Overloaded immunoprecipitation following procedure observed in Figure 15B. IgG is observed at approximately 50 kDa and GAPDH is observed at approximately 37 kDa.

Densitometry

To assess potential differences in CRH expression between agonist and antagonist treated specimens, we used FIJI to analyze the optical density of the bands from the immunoprecipitations of Figure 15B (Figure 17). Because of loading problems in Figure 16, only Figure 15B was analyzed. Since the same dilution of IgG was used for all samples, the optical density of IgG bands was used as a reference for comparison to CRH bands by generating a ratio. Differences in optical density were observed. CRH levels in agonist treated specimens was lower than untreated controls and antagonist treated specimens. CRH levels in antagonist controls were higher than untreated controls and agonist treated specimens. DMSO specimens had the lowest level of CRH protein. It should be noted these are preliminary results from a single experiment;

therefore, due to lack of replications, significance cannot be obtained from this data. The differences observed, however, warrant additional testing.

	Control	DMSO	Ag	Ant
IgG	134875	137824	117106	131966
CRH	48126	27248	26561	64853
Ratio CRH:IgG	0.35681928	0.19770142	0.22681161	0.49143719

Figure 17. FIJI densitometry analysis of observed bands from immunoprecipitation in Figu15B. Numbers represent FIJI's measurement of optical density based on pixel intensity.

DISCUSSION

This study aimed to investigate the potential role 5-HT plays in modulating the anxiety pathway, the effects of increased or decreased 5-HT activity on anxiety behavior and, more specifically, how its activity via the 5-HT7 receptor subtype affects production of the cortisol activation pathway precursor, corticotropin releasing hormone. Anxiety behavior was assessed based on measured distance moved after a sound stimulus with greater movement indicating more anxiety and protein detection CRH production was assessed by immunoprecipitation combined with SDS-PAGE and silver stain after it was determined that Western blot analysis would not provide the level of sensitivity needed to detect protein in the experimental conditions used for this study. Although preliminary results were not sufficient for analysis of statistical significance and could not be reproduced because of time constraints, the data obtained warrants future study.

Behavioral Implication of 5-HT7R Agonist and Antagonist on Anxiety

Behavioral responses to auditory stimulus suggest anxiety behavior in zebrafish larvae is affected by changes in 5-HT activity (Figure 13). When comparing the distance traveled in response to the sound stimulus for agonist treated and antagonist treated larvae, there is no notable difference 15 minutes after treatment, but there is a statistically significant difference 22 hours after treatment. The difference detected is consistent with the hypothesis that increased 5-HT activity mimicked by the agonist would result in lower anxiety levels and decreased 5-HT activity mimicked by the antagonist would result in higher anxiety levels. However, both agonist and antagonist treated larvae exhibited less distance moved than untreated control larvae. While this might be expected for agonist treated it was not expected for antagonist treated larvae. Recognizing that the DMSO solvent used to reconstitute the agonist and antagonist may have an effect on the larvae, a DMSO only treatment control was included in the behavioral experiments. However, no movement data were collected for DMSO because they died upon treatment. This was an unexpected result because the concentration of DMSO used was the same concentration of DMSO present in the agonist and antagonist treatments. The apparent lethality of DMSO only treatment may be explained by the source of DMSO used. There were two bottles of DMSO available, and it is possible that one bottle had been used to reconstitute the agonist and antagonist and, when that bottle had run out, the other, older bottle had been used for the behavioral assays. Because of this, the data observed here may only be considered as preliminary and further testing is required to assess effects of DMSO on behavior before conclusions can be drawn.

CRH Production Analysis on 5dpf Larvae

Western Blots

Initially, fluorescent Western blot analysis was chosen as a method for detecting protein so that we could visualize both the target protein, CRH, and a constitutively expressed control protein, GAPDH, simultaneously from the same specimens. Since GAPDH is an enzyme involved in a basic metabolic pathway that is common to all living cells, the amount of it present should not be affected by the agonist and antagonist treatment. Therefore, GAPDH protein levels can serve as a control by which changes in CRH protein level can be compared.

For initial experiment undertaken to optimize the method, due to short shelf-life of agonist and antagonist once reconstituted, only wild type larvae were used. Initial failure to detect protein with fluorescence pushed us to examine potential variables which may have affected the procedure. We tested several different theories as to why we didn't detect protein, including fluorescent antibody dot blot tests, concentration of loaded protein, and frozen versus fresh protein extract. The UVP gel imaging system is equipped with filters to detect fluorophores in the green and red range. To ensure the fluorescently tagged antibodies could be detected by the imaging system, we added each antibody to a dry PVDF membrane and viewed in the imager. Fluorescence was detected, confirming we were using the correct fluorophores. Subsequently, we analyzed several PVDF membrane treatment conditions used in different protocols for immunoblotting. These treatments included methanol rinse before antibody addition, TBST rinse before antibody addition, and allowing membrane to dry then TBST rinse before antibody addition. The second method produced the best results for dot blots (Figure 14). This method was used for all following Western blots. We also tried using several dilutions of protein to sample buffer, including 1:1000, 1:100, 1:10, and pure protein extract. No dilution

resulted in presentation of bands. Finally, comparison of fresh versus frozen protein extracts resulted in no detection of bands. Repeated failure to detect protein using the fluorescence western blotting technique led us to believe that the level of sensitivity of this method was not sufficient to detect CRH or GAPDH in our samples.

Immunoprecipitations

Since the need for a protein detection method that was more sensitive than fluorescent Western blot was apparent, we decided to use immunoprecipitation combined with SDS-PAGE and silver stain. The initial immunoprecipitation protocol needed to be optimized for our experiments as the protocol indicated several time points at which samples could be frozen. We attempted the protocol several times with freezing following protein extraction. Freezing following immunoprecipitation, and with fresh extract. Results indicated that using fresh protein extract led to the most visible bands during SDS-PAGE. However, because the process was rather lengthy, it was difficult to complete it with the most optimized protocol within the time constraints. Furthermore, because the protocol required the use of fresh protein extract in order to detect proteins, it was not possible to use the frozen extracts obtained from specimen used in the behavioral assays. Due to time constraints, we were unable to complete treatments with behavioral analysis followed by immunoprecipitation. We were also unable to complete all planned immunoprecipitations, so the data presented in this paper are very preliminary. However, results from one completed immunoprecipitation analyzed by densitometry indicate the hypothesized decrease in CRH levels in agonist treated larvae and increase in CRH levels for antagonist treated larvae.

Due to problems with the DMSO control data, these comparisons only provide preliminary evidence that 5-HT may be functioning as we hypothesized and more strongly necessitates the need for continued research. Furthermore, the differences in simultaneous detection of CRH and GAPDH indicate further optimization may be needed to improve the efficacy of the current procedure. In addition, immunoprecipitations should be completed on specimens that have undergone behavioral analysis to provide more accurate data on the effect of treatment on 5dpf larvae. Finally, use of densitometry data should be done as well to quantify differences in optical density of the observed bands for each treatment.

Future Research and Implications

While this project produced no decisive conclusion of 5-HT effect on production of CRH and anxiety behavior, some evidence from the agonist and antagonist treated specimen does suggest 5-HT functions in the way hypothesized. This study provides preliminary data that 5-HT activity via the 5-HT7 receptor subtype may have some modulatory effect on production of CRH and anxiety behavior, but further testing is required to come to a decisive conclusion. With this in mind, research to assess the role which 5-HT plays in the anxiety pathway should continue. Furthermore, should these preliminary results be confirmed, expansion of this project into other functions affected by ASC would be called for to determine the role which 5-HT may play in the production of these symptoms. Areas for future research could include 5-HT functioning in the gastrointestinal tract, 5-HT modulation of sleep behaviors, and assessment of the role 5-HT plays in modulating social behaviors that lead to social dysfunction.

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APPENDIX

Appendix I: Camera Procedure

Camera Set Up and Filming Procedure

1. Plug in computer cart to wall
2. Plug camera into wall - the camera port is on the upper right side of the camera when looking through the eyepiece, under a flap
3. Connect camera to computer via firewire - camera port is under a flap on the bottom next to the screen and the computer port is on the left side
4. Open iMovie on computer
5. In iMovie, right click on left side, click create new event
6. Click import under new event
7. Click Sony camera
8. On camera, click record
9. In iMovie, click import on bottom right hand of screen to start recording
10. Click Stop import in iMovie to stop recording
11. Click stop recording on camera
12. Ex out of iMovie import screen
13. Click folder that film was recorded too
14. Edit movie if necessary
15. Click share in top right corner of screen to file
16. Save movie
17. Upload to OneDrive and email to computer with Fiji application

To Rewind Camera

1. Use on/off switch to switch camera setting to play/edit
2. Click rewind on lower left corner of screen
3. Rewind to 00:00:00
4. Switch back to tape
5. Continue recording

Appendix II: Western Blot and BCA Procedure

Western Blot Procedure

Protein Extraction

1. Make 2x RIPA Buffer, 2x Protease Inhibitor, 5x Sample Buffer, and 10x Running Buffer before beginning process (See recipe list). Put homogenizer on ice. Begin heating block to 95 degree C if using protein extract immediately
2. Add Tricaine to petri dish w/ larvae (see tricaine procedure on wall)
3. Transfer larvae to eppendorf tube and remove excess E3 buffer
4. Mix 70 microL Protease Inhibitor and 70 microL 2x RIPA buffer to separate eppendorf tube (for 30 bros)
5. Add ~120 microL of RIPA mixture to eppendorf containing larvae
6. Remove larvae and RIPA mixture and add to homogenizer on ice
7. Homogenize larvae
8. Remove homogenized larvae and place in eppendorf
9. Centrifuge for 30 minutes at 13000 G at 4 degree C
10. Remove supernatant and place in new eppendorf tube. This can either be used immediately or stored at -80 degrees C

Gel Electrophoresis

1. Before beginning, pull gel out to thaw, pull out sample buffer to thaw, heat block to 95 degrees C, make 1x Running Buffer from 10x stock
2. Make specific protein extract concentrations. For this experiment:
    a. 10 microL milliQ, 5 microL sample buffer, and 5 microL protein extract

b. 5 microL milliQ, 5 microL sample buffer, and 10 microL protein extract

c. 5 microL sample buffer and 15 microL protein extract

3. Pipette 10 microL of each concentration into different aliquots, label, and heat at 95 degrees C for 5 minutes
4. While tubes are heating, open gel and rip off bottom sticker. Place in gel box with clips near top on either side.
5. Pour 1x Running buffer between wall and gel, as well as on the bottom
6. Quickly centrifuge tubes after heating
7. Pipette contents of each tube into separate wells of gel
8. Plug machine in and turn to volts. Plug cap onto set up and machine (black to black, red to red). Set to ~106 volts (can be changed with center dial). Turn on machine and run for ~1 hour. Gel can be stored at 4 degrees C for ~12 hours.

Transfer

1. Make Transfer Buffer and fill Transfer box with Transfer Buffer
2. Open transfer mechanism (white slider) and lay open.
3. Dunk sponge in transfer buffer and place on open mechanism. Then dunk transfer paper and place on mechanism
4. Cut membrane from roll w/ gloved hands to approximate size of gel (touch as little as possible). Dunk membrane and remove paper on either side of membrane. Place on open mechanism and roll out air bubbles with pipette tip

5. Remove gel from case by running scoopula between plastic until it cracks off. Place gel on saran wrap. Use razor to cut off wells. Place gel on open mechanism.
6. Dunk second transfer paper and place on mechanism. Then dunk second sponge and place on mechanism. Close mechanism and place in transfer box.
7. Turn on machine (like running a gel) and run for ~1 hour
8. Once finished, remove and throw away gel
9. Make block solution. Move transfer paper to tupperware container and pour Blocking Solution over transfer paper until it is covered. Place in cold room on rocker for ~2 hours. Can be stored in 1x TBS at 4 degrees C.

Immunohistochemistry

1. Block blots in blocking solution for 1-2 hours at RT on shaker
2. Add primary antibody diluted in block solution
3. Incubate overnight at 4 degrees C on shaker
4. Rinse with TBST (make fresh) 3x 5 minutes at RT
5. Add secondary antibody diluted in either block solution or TBST
6. Incubate for 1 hour at RT. Cover if using fluorescently tagged antibody.
7. Rinse with TBST 3x 10 minutes at RT. Cover if using fluorescently tagged antibody.
8. Image immediately

BCA Protein Kit Assay

Preparation of Standards

1. Prepare diluent - equal parts 2x Protease Inhibitor and RIPA buffer

2. Break ampoule - file around etching, get all liquid to bottom, hold between thumbs and forefingers with forefingers touching, break towards you
3. Pipette according to Table 1 making sure to minimize bubbles
    a. Be sure to mix dilutions before taking from them for further dilutions

Table 1. Preparation of standards

Tubes	**Volume of Diluent (microL)**	**Volume and Source of BSA (microL)**	**Final BSA Concentration (microg/mL)**
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of tube B	750
E	325	325 of tube C	500
F	325	325 of tube E	250
G	325	325 of tube F	125
H	400	100 of tube G	25
I	400	0	0 = blank

Preparation of Working Reagent

1. Determine total volume required

   (# of standards + # of unknowns) x (# of replicates) x (volume per sample) = total volume WR required

2. Mix 50 parts Reagent A with 1 part Reagent B

Preparation of Sample Diluents

1. Mix 1 part sample with 100 parts of 2x Protease Inhibitor and RIPA solution
2. Mix 1 part sample with 10 parts of 2x Protease Inhibitor and RIPA solution

Microplate Procedure

1. Pipette 10 microL of standard or sample into individual wells according to Table 2

Blank	A	B	C	D	E	F	G	H	X1	X2	X3
Blank	A	B	C	D	E	F	G	H	X1	X2	X3

2. Add 200 microL of WR to each well being careful to minimize bubbles
3. Place film on top and mix on plate shaker for 30s
4. Incubate at 37 C for 30 min

a. Incubation time can be increased to up to 2 hours to increase sensitivity, if necessary

5. Cool plate to RT and place in microplate reader
6. Save run to USB in .xlsx format and analyze in excel

Appendix III: Immunoprecipitation Procedures

Immunoprecipitation

Protein Extraction

11. Make 2x RIPA Buffer, 2x Protease Inhibitor, 5x Sample Buffer, and 10x Running Buffer before beginning process (See recipe list). Put homogenizer on ice. Begin heating block to 95 degree C if using protein extract immediately
12. Add Tricaine to petri dish w/ larvae (see tricaine procedure on wall)
13. Transfer larvae to eppendorf tube and remove excess E3 buffer
14. Mix 70 microL Protease Inhibitor and 70 microL 2x RIPA buffer to separate eppendorf tube (for 30 bros)
15. Add ~120 microL of RIPA mixture to eppendorf containing larvae
16. Remove larvae and RIPA mixture and add to homogenizer on ice
17. Homogenize larvae
18. Remove homogenized larvae and place in eppendorf
19. Centrifuge for 30 minutes at 13000 G at 4 degree C
20. Remove supernatant and place in new eppendorf tube. This can either be used immediately or stored at -80 degrees C

Immunoprecipitation

1. Fresh or thawed supernatant should be aliquoted into an Eppendorf tube at 100 ul and add ***concentration of antibodies*** were to the sample
2. Add 30 ul of 1:1 slurry of protein-A sepharose beads in NET buffer to sample
    a. Bead slurry should be made before immunoprecipitation is begun
        i. Swell needed amount of beads in NET buffer on ice for 45 min-2h, until beads settle
        ii. Pipette off excess buffer and add fresh buffer at the original volume. Allow beads to settle. Keep on ice.
        iii. Pipette off excess buffer and add NET buffer at a volume equal to the settled bead bed. Keep on ice. Bead slurry can be kept at 4ºC for one week
3. Centrifuge immunoprecipitation samples 1 min
4. Aspirate buffer
5. Wash with 1 ml cold NET buffer
6. Repeat steps 3-5
7. Repeat steps 3 and 4, wash with 1 ml cold 0.01 M Tris, pH 6.7
8. Centrifuge samples for 1 min
9. Aspirate buffer
10. Add 30 ul running buffer and either use immediately for SDS-PAGE or store at -20ºC

SDS-PAGE

1. Add 1x Running Buffer to gel apparatus
2. Pipette 11 ul of Marker and samples into individual wells of SDS-PAGE gel
3. Run at 106 volts for one hour

4. Remove gel from case and immediately begin silver stain procedure

Silver Stain

1. Immerse gel in 100 ml Fixative, place on a shaker at 60-70 rpm for 20 min
    a. Prepare Fixative by mixing 50 ml ethanol with 10 ml glacial acetic acid and 40 ml deionized water
2. Wash gel in 100 ml 30% ethanol, place on shaker at 60-70 rpm for 10 min
    a. Prepare 30% ethanol by mixing 30 ml ethanol with 70 ml deionized water
3. Wash gel in 100 ml deionized water, shake at 60-70 rpm for 10 min
4. Incubate gel in 100 ml Silver Stain Sensitizer Working Solution
    a. Prepare Silver Stain Sensitizer Working Solution by mixing 1 ml Silver stain Sensitizer with 99 ml deionized water. Use within two hours after preparation
5. Wash gel in 200 ml deionized water and shake at 60-70 rpm for 1 minute. Replace water and shake for an additional minute
6. Incubate gel in 100 ml Silver Stain Working Solution and shake at 60-70 rpm for 20 minutes
    a. Prepare Silver Stain Working Solution by adding 1 ml Silver Stain with 99 ml deionized water. Use within two hours after preparation.
7. Wash gel in 100 ml deionized water and shake at 60-70 rpm for 30 seconds. Replace water and shake for an addition 30 seconds
8. Add 100 ml Developer Working Solution and shake at 60-70 rpm until protein bands appear (~3-10 minutes)

a. Prepare Developer Working Solution by adding 20 ml Silver Stain Developer B into 80 ml deionized water and 0.05 ml of Silver Stain Developer A

9. When desired band intensity is achieved, replace Developer Working Solution with Stop Solution. Shake at 60-70 rpm for 10 minutes
    a. Prepare stop solution by mixing 5 ml glacial acetic acid with 95 ml deionized water
10. Replace Stop Solution with deionized water. Shake at 60-70 rpm for 2-5 minutes
11. Image immediately and store at 4^{o}C

UVP Imaging System Set Up and Image Processing Protocol

1. Biolite source set up

Bulb - redlight

Intensity - max at 6

Filter Position - 2, blue light

1, green light

Fiber Optic light - Epi

Emission Filter - 1 w/ green light

2 w/ blue light

2. Acquisition set up

Lens:

Aperture - 2.1

Zoom - ~25

Focus - far side

Max out gain in preview window

Capture image

3.Image Processing Set Up

Under Image tab

Histogram

Stretch mode to ‘Automatic’

Corrections

Brightness - 15

Contrast - 60

Gamma - 1.9

Densitometry Analysis Procedure

Piasecki, Cell Bio

1. Open immunoblot image: file → open → immunoblot.tif
2. Convert RGB image to an 8-bit B/W image so that you can quantify pixel intensity (image → type → 8-bit)
3. Subtract the background (Process → subtract background) using light background at 50 pixel radius
4. Invert the image to make protein light (higher pixel values) and background dark (lower pixel values) – (Edit → Invert)
5. Make sure the measurement “integrated density is selected” (Analyze → Set Measurements → Integrated Density)
6. Use box tool to select and measure both background and protein bands.
    a. Select the box tool from the clipboard (Far left)

    b. Using your mouse select a region surrounding the largest protein band.
    c. Using the keyboard press command M (alternatively select Analyze → Measure)

d. Using your cursor, select *inside* the yellow square and move the box to a new region of the image; press command M.
e. Repeat the previous step until the integrated density of both the top and bottom bands of each of the three wells, as well as two or three background values, have been analyzed.
f. Place this information into a spreadsheet, subtract the average background band from each of your individual top and bottom band measurements, and determine the ratio of the top and bottom bands from each background corrected value.