SEROTONERGIC MODULATION OF MAUTHNER NEURONS IN LARVAL ZEBRAFISH

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By

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Abstract

Serotonergic Modulation of Mauthner Neurons on Larval Zebrafish

Tyler Chase Purvis

In recent decades, zebrafish have become a popular organism in neuroscience research. This is primarily because of the homogenous nature of what are known as reticulospinal neurons. These neurons, located in the hindbrain, control locomotive (e.g., swimming) and escape behaviors via descending axonal connections to lateral musculature. Dominating the reticulospinal neuron group are the Mauthner neurons. This pair of morphologically distinctive neurons is referred to as “command neurons” in the reticulospinal group. Recent studies involving antibody labeling reveal a distinctive clustering of serotonin (5-HT) associated proteins in the Mauthner dendritic regions. This would imply that 5-HT plays a direct role in the modulation of Mauthner neuron activity.

In this study, we attempt to experimentally demonstrate a functional relationship between 5-HT and the Mauthner neuron through the administration of the SSRI fluoxetine and Ca²⁺ imaging techniques. Results indicate no significant change Ca²⁺ and latency of response. Interestingly, these data indicate a difference in responsiveness (i.e., firing or no firing) with the fluoxetine group showing an 8% increase. These data shed light upon the Mauthner neuron and 5-HT, as well as the possible considerations of future research.
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Introduction and Literature Review

The study of lower organisms has greatly advanced neurobiology (Carew, Walters, & Kandel, 1981). In the last thirty years, zebrafish, (*Danio rerio*) have become an organism at the forefront of developmental genetics, neuroscience, and psychobiology (Barros, Alderton, Reynolds, Roach, & Berghmas, 2008). This is especially true for the field of neuroscience, where in recent years the zebrafish nervous system has received increasing attention and experimentation (see Figure 1).

*Figure 1*. Publications on PubMed using the search terms zebrafish and neuroscience. The trend indicates that in the last thirty years zebrafish research has steadily increased.
Larval zebrafish nervous system has been extensively researched and documented (Barros et al., 2008). This fact extends to a cellular and molecular level. Many individual zebrafish neurons have been catalogued in terms of location, orientation, and both afferent and efferent synaptic connections. The zebrafish nervous system is nearly identical in structure and composition across individual animals which permit the experimentation of a catalogued neuron or neural network across many fish (Eaton, Lee, & Foreman, 2001). This homogeneous neural network allows for the acquisition of large amounts of data concerning specific experimental interests across fish populations, and is arguably the greatest strength of neural zebrafish research.

Like all fish, zebrafish are vertebrates. This places zebrafish much closer to humans (Homo sapiens) upon the evolutionary ladder than some of the more traditionally examined organisms in neuroscience, such as sea slugs, leeches, and earthworms (Thomas, Mario, & Wullimann, 2002). In the past, common invertebrates (e.g., earthworms) have been often utilized in neuroscience research because of the relatively simple nature of their nervous systems (Sandeman, 1999). Species with a simple nervous system typically execute stereotypical motor behaviors. Simple motor behaviors, mediated by straightforward neural circuitry, makes the causal relationship between stimulus and response more easily demonstrated and understood.

Reductionism, or the breaking of complex problems into smaller, easier to resolve segments, is an established strategy in science. Human biological psychology demands a reductionism strategy, given the vast intricacies and overall complexity of the human central nervous system. Zebrafish are an excellent means of employing such a strategy,
given their evolutionary relationship as vertebrates and the relative simplicity of their nervous system. As implied, simple organisms can vastly increase knowledge of the underlying mechanisms involved with brain function (Llinas, 2002). For instance, Kandel’s famous learning experiments using *Aplysia californica* revealed a neurochemical explanation regarding the well-established phenomena of classical conditioning (Carew et al., 1981). In this particular case, the anatomy and circuitry of neurons that facilitate learning in sea slugs proved to be a relevant template for homologous functions in more complex animals including humans. Species specific behaviors, such as the gill withdrawal response (i.e., retraction of the siphon/gill of the sea slug when disturbed) in *Aplysia californica* are typically well defined and require simple sensorimotor neuronal circuitry (Carew et al., 1981). However, it is important to keep in mind what the word simple refers to, relative to the complex field of neuroscience. A simple vertebrate neuronal system could range from 50,000 neurons as in zebrafish, to many millions as in the embryonic rat brain. When confronted with such overwhelming complexity, it’s easy to understand why the study of lower life forms is essential in understanding the neurobiological bases of human behavior. Zebrafish are an essential part of this effort, and will undoubtedly continue contribute to our overall understanding of sensorimotor circuitry in humans.

**Sensorimotor Systems**

One of the core functions of any nervous system is to extract relevant information from sensory inputs and use this information to guide appropriate motor behaviors. This
type of neural activity is referred to as sensorimotor processing (Bedau, 1994). Much of the brain is involved with this task, so it comes at no surprise that this topic has been extensively researched in humans and many other organisms (Massino & Fetcho, 2005; Mclean & Fetcho, 2004a; Oda, 2008).

The aim of many neurobiologists is to comprehend how neuronal population activity relates to sensory processing and control of behavioral responses (Goulding, 2009). This endeavor is challenging for the simple fact that even relatively simple behaviors arise from different levels of neural organizational throughout the nervous system (McLean & Fetcho, 2004a). Due to advances in optical, electrophysiological and genetic research techniques, the larval zebrafish has become an invaluable tool for the investigation of the mechanisms involved in generating patterned behavior in response to sensory stimulation (Massino & Fetcho, 2005). These species specific behaviors found in biological organisms have evolved to solve particular evolutionary challenges. The sensorimotor system utilized in aquatic and land based organisms is critical for survival, for this system enables a creature to sense and react to potentially life threatening situations (e.g., sensory evoked escape maneuver in fish and amphibians; Oda, 2008). In mammals, primary sensorimotor processing is controlled by the cerebral cortex. Rather, zebrafish sensorimotor processing is achieved via less intricate pathways originating in the hindbrain. This makes larval zebrafish an ideal candidate for the study of the hindbrain sensory systems in which all vertebrate species share.

Reticulospinal neurons located in the zebrafish hindbrain give rise to the reticulospinal tract, an evolutionarily conserved descending motor pathway that exerts
control over movements in vertebrates (Riddle, Edgley, & Baker, 2009). The term “evolutionarily conserved” in this context refers to the fact that reticulospinal neurons are found in all vertebrates, from primitive lower vertebrates like lamprey (*Petromyzon marinus*) to higher order mammals like chimpanzees and human beings.

Over the course of millions of years, mammals have evolved a dominant descending motor pathway known as the corticospinal tract, which originates in layer V of the neocortex (Kuang, Merline, & Kalil, 1994). The reticulospinal and corticospinal tracts function to coordinate movement, the primary difference being the latter facilitates a higher degree of control and is associated with voluntary movements (Riddle et al., 2009; see Figure 2). Moreover, although all mammals utilize the corticospinal tract for voluntary movement, retention of the older and inferior reticulospinal tract has occurred across species. This suggests that experimental research involving this pathway could providing a knowledge base for future study of more advanced means of motor processing.

**Reticulospinal Neurons**

Fish are some of the fastest moving animals on Earth, and their physical speed is dependent upon the precise and sequential activation of reticulospinal neurons and their subsequent musculature (Gleason et al., 2004). This speed is profoundly exhibited in escape movements. Escape movements and spontaneous swimming circuitry develops rapidly in zebrafish. Tactile evoked movements can be seen in zebrafish as early one day post fertilization (1dpf), while full escape responses can be observed at 2dpf (Gleason et
Figure 2. Comparison of the reticulospinal and corticospinal descending motor tracts. The corticospinal tract (right) originates in layer V of the neocortex while the reticulospinal tract (left) originates in the brainstem. The corticospinal tract exerts greater control over movement.

al., 2004). Around 4dpf spontaneous swimming is first observed. This apparent rapid development escape behaviors and motor innervations speak to the overall importance of such behaviors.

As stated, the escape response, also known as the C-bend, is arguably the most important behavior exhibited by fish (Oda, 2008). This behavior re-orient the fish to quickly evade a predatory threat via isolated contortion of stimulus relevant ipsilateral
musculature in unison with contra-lateral inhibition. The behavior is observed in fish and amphibians species alike (Sillar, 2009). The escape response is mediated by reticulospinal neurons, which directly synapse upon spinal inter neurons that drive motor activation. This particular behavior, is one the primary duties of reticulospinal neurons.

Reticulospinal neurons are categorized as inter-neurons with descending spinal axons and cell bodies located in the hindbrain (Duboc et al., 2008; Soffie, Roberts, & Li, 2009). Reticulospinal neurons are the intermediary between the brain and spinal cord, and function to ensure that the animal executes the correct (i.e., adaptive) response, given the nature of the sensory stimulus (Soffie et al., 2009; see Figure 3).

It is widely accepted that reticulospinal neurons operate to provide the necessary commands to locomotor circuitry located in the spinal cord (Soffie et al., 2009). Research involving reticulospinal neurons in lampreys, a primitive jawless fish, indicate the unique ability of reticulospinal neurons to transform short interval excitatory stimuli into long duration excitatory drive of spinal locomotor circuits (Duboc et al., 2008). Larval zebrafish reticulospinal neurons provide a unique opportunity to investigate in further detail how patterns of neuron activity influence spinal locomotive circuitry (Masino & Fetcho, 2005).

Artificial electrode stimulation of reticulospinal neurons evokes the initiation of swimming behaviors in Xenopus tadpoles (Soffie et al., 2009). Similarly, intra-cellular reticulospinal recordings during spontaneous fictive swimming, a procedure in which the nervous system is active but the specimen is paralyzed in tadpoles reveal reticulospinal neurons firing in unison with observed swimming behaviors (Soffie et al., 2009).
Figure 3. The reticulospinal group. This image of the zebrafish hindbrain, includes the nucMlf neurons which receive sensory information from the optic tectum, the M-series group, descending reticulospinal axons, and the locations where the Mauthner neurons would be found had they been labeled with a Ca$^{2+}$ indicator. Also depicted are the partial outlines of the left and right eyes.
Central Pattern Generation and Spinal Locomotion Circuitry

The nervous systems of many organisms contain what are referred to as central pattern generating (CPG) circuits. These intrinsic neuronal circuitries produce stable, stereotypical rhythmic firing patterns and well defined motor output (Kiehn et al., 2008). CPG circuits are involved with a number of physiological functions, including respiration, feeding, and locomotion (Marder & Rehm, 2005). From an evolutionary standpoint, the integrity of CPG circuitry is paramount for survival. For example, in order for an organism to survive early stages of development it is vital that respiration CPG circuitry develops rapidly and correctly.

CPG spinal locomotor activity begins with reticulospinal neuron activity (Gabriel et al., 2008). CPG circuitry exerts controls the over musculature involved in locomotion through the regulated output of spinal motor neuron axons onto peripheral muscle cells (Kiehn, 2006). Because locomotion in organisms requires stereotypical patterns of activity, spinal networks have evolved the necessary CPG circuitry to mediate the required action. In other words, the intrinsic architecture of CPG circuitry is genetically hard wired to produce a prescribed rhythmic output upon command (i.e., reticulospinal descending input).

Scientists have long been interested in how exactly neuron circuitry develops and organizes to produce the patterned outputs that underlie motor behaviors (Goulding, 2009). In larval zebrafish, locomotion begins at approximately 18 hours post fertilization with rudimentary tail flips. At 28 hours post fertilization the escape response is first
observed, and finally at 4-5dpf spontaneous swimming occurs (Thirumalai & Cline, 2008). These developmental milestones are the cumulative effect of CPG and reticulospinal neuron maturation.

**Mauthner Neurons and Homologs**

There are approximately 240 reticulospinal neurons in the zebrafish hindbrain. These reticulospinal neurons are grouped into seven distinct clusters projecting along rostrocaudal axis (Oda, 2008). At the center of the reticulospinal system lie the Mauthner neurons, a giant pair of neurons that project contralaterally to CPG neurons located in the spinal cord. Laterally and ventrally projecting Mauthner dendrites receive retinal, and lateral line inputs (Mclean & Fetcho, 2004a). Mauthner neurons represent a crucial link between vertebrate and invertebrate research in neuroscience. Eaton, Lee, and Forman (2001) assert that Mauthner network functionality may provide important clues about the network function of more recently evolved neural circuitry.

Mauthner neurons have been empirically shown to mediate the fast escape response in zebrafish (Mclean & Fetcho, 2004a). Research indicates that ablation (i.e., destroying) of Mauthner neurons increases latency of the escape response (Kohashi & Oda, 2008). It is interesting to note that the key word is “latency” and not “elimination” of the escape response. This indicates that the Mauthner neurons are not the sole reticulospinal neurons involved in this response. Nonetheless, Mauthner neurons are of great importance, and may serve as a “command neuron” for the fastest escape turns in lower vertebrates (Eaton et al., 2001).
Mauthner neurons belong to a group of reticulospinal neurons referred to as the M-series, which include neurons, Mid2cm and Mid3cm (Kahashi & Oda, 2008; see Figure 4).

Figure 4. The Mauthner Neuron and homologs. The Mauthner cells are morphologically distinct, consisting of large cell bodies and highly visible bilaterally crossing descending axons.

These neurons are morphologically similar to the Mauthner neuron, in that they contain laterally projecting dendrites and contralateral spinal axons. The M-series neurons are theorized to collectively mediate the “escape response” reticulospinal circuit. An ablation study conducted by Lui and Fetcho (1999) found that destruction of Mauthner neurons caused a cessation of fast escape responses from tail stimulation, while
ablation of all M-series cells eliminated fast escape responses from both head and tail stimuli. Evidence of this nature indicates that not only do morphologically similar reticulospinal neurons operate in function groups, but that they respond to specific stimuli (i.e., stimulus directionality).

**Serotonin and Reticulospinal Neurons**

Five-hydroxytryptamine (5-HT), more commonly known as serotonin, is a monoamine neurotransmitter found in the central/peripheral nervous systems. 5-HT plays a role in the modulation of spinal locomotor circuitry (Gabriel et al., 2009). Research suggests that 5-HT modulates the spinal network activity of all vertebrates (Gabriel, et al., 2009). In rats and mice, previous work has demonstrated that bath application (i.e., complete liquid immersion) of 5-HT activates spinal locomotor circuitry (Madgriaga, McPhee, Chersa, Christie, & Whelan, 2004). Studies involving 5-HT and locomotion in mammals are a welcome rarity, and strengthen the argument that 5-HT may modulate the spinal networks of all vertebrates.

Research involving the modulation of locomotor activity by 5-HT typically revolves around three aquatic organisms; lampreys, *Xenopus* tadpoles, and zebrafish. Researches involving these three species have consistently demonstrated 5-HT modulation of locomotor behaviors. However, these results differ between species. For example, 5-HT has been shown to decrease swimming frequencies in lamprey, yet increases the intensity and duration of swimming frequencies in *Xenopus* (McDearmid, Scrymgeour-Wedderburn, & Sillar, 1997). In zebrafish, the effect of 5-HT seems to be
developmentally dependent. The application of exogenous 5-HT (how) in zebrafish has been demonstrated to increase the burst frequency of spontaneous swimming episodes in larvae, and similar effects have been observed using the 5-HT agonist quipazine (Brustein et al., 2003).

5-HT appears to modulate larval zebrafish spinal network activity by altering periods of inactivity between swim bursts. Brustein (2003) demonstrated this effect using the 5-HT antagonists’ methysergide and ketanserin. In this study the applications of 5-HT antagonists were shown to decreases swim burst frequencies via extension of inactivity periods while the opposite is observed for agonists (i.e., reduction of inactivity periods).

5-HT modulation of zebrafish swimming behaviors first occurs at approximately 4dpf, which coincides with the onset of spontaneous swimming behaviors (Brustein et al., 2003). Work by McLean and Fetcho (2004b) supports these findings, showing that 5-HT antibodies in the zebrafish spinal cord are first observed at 4dpf. The development of 5-HT neurotransmission in the spinal cord at 4dpf and simultaneous occurrence of spontaneous swim activity in larval zebrafish has led scientists to postulate that the role of 5-HT in larval zebrafish may be to increase locomotor output (Brustein et al., 2003).

The underlying mechanism by which 5-HT modulates spinal activity is somewhat elusive. However, a functional relationship appears to exist between 5-HT and the primary excitatory neurotransmitter amino acid glutamate. 5-HT is known to depress glutamtergic transmission in the spinal cord (Shupliakov, Pieribone, Gad, & Brodin, 1995). Previous research found that 5-HT reduced NMDA (a glutamate agonist) induced fictive swimming behaviors in zebrafish (Gabriel et al., 2009). It is also important to note
that this particular study involved juvenile and adult zebrafish, and there exists a disparity between the effects of 5-HT in larval and adult zebrafish (Gabriel et al., 2009). In adult zebrafish the application of 5-HT appears to decrease the frequency of fictive swimming behaviors. This is in direct contrast to observations of zebrafish larvae. It is also important note to that the means by which swimming frequencies are altered varies between larvae and adults. In larvae, the quiescent period (i.e., rest time) between swim bouts is decreased by 5-HT, while in adults the burst frequency is increased with no alteration of rest intervals (Gabriel et al., 2009). The researchers suggest that future studies should attempt to uncover the molecular/genetic aspects involved with this “switch” of the 5-HT effect.

Previous research studies have shown that NMDA can induce motor activity in the lamprey, *Xenopus*, neonatal rats, rabbits, and felines (Dale & Roberts, 1989; Fenaux et al., 1987; Grillner et al., 1981; Kudo & Yamada, 1987). Recently it has been shown in rats and humans that 5-HT and glutamate receptors form complexes in the postsynaptic membrane, yielding a receptor with distinct properties that influence motor activity. Combined with 5-HT research, a relationship between glutamate and 5-HT in the modulation of spinal circuitry suggests an across species pharmacological method of locomotive modulation. Research involving the lamprey may reveal how this relationship may work. Using lampreys, Shupliakov et al. (1995) demonstrated that the number of synaptic vesicles containing glutamate were three times higher in reticulospinal axons exposed to 5-HT than without exposure, indicating that 5-HT reduces the rate of synaptic exocytosis
**Calcium Imaging and Laser Confocal Microscopy**

An advance in biotechnology now allows for real time precision imaging of neuronal populations. Through such methods, intricate and distinct neuron populations in various organisms can effectively be imaged, and their cellular responses to stimuli can be recorded (Ritter, Dimple, & Fetcho, 2001). Calcium imaging (CI) via laser confocal microscopy (LCM) is one the most common and effective methods utilized in the real time visualization of neurophysiology (Hagashima, Masino, & Mandal, 2003). This is achieved through the use of calcium indicators, which are fluorescent molecules that bind to Ca²⁺. LCM allows the researcher to observe in situ the subsequent nervous system responses to stimuli. Zebrafish are especially suited for LCM research. In their larval stage, zebrafish are relatively transparent, thus increasing microscope laser penetration and overall tissue resolution (Hagashima et al., 2003).

Through LCM, it is now possible to image neurons and glia cells up to 500μm below a tissues surface in vivo (Garaschuk, Milos & Konnerth, 2006). This is very different than staining of various cortical brain tissues and sub components. Such methods utilize ex vivo methods and do not indicate neuronal activity. LCM, however, allows researchers to accomplish this difficult task (i.e., in situ recordings).

One of the strongest advantages of LCM is the ability to produce image stacks, that is, to automatically acquire multiple focused sequential images with varying degrees of tissue depth (Ritter et al., 2001). This technique is known as optical sectioning, and allows for the visualization of three dimensional neuron populations. The ability to make
image stacks is especially important in research involving dual labeling techniques, such as immune-labeling and calcium backfilling. For research involving neuron populations, image stacks can also serve as an anatomical reference micrograph (see Figure 5).

*Figure 5.* Example of optical sectioning in laser confocal microscopy. The dotted lines (*Lines 1-3, top to bottom respectively*) drawn through the zebrafish head represent varying levels of depth.
Populations of neurons are rarely found upon the same plane of depth. Instead, populations of neurons often have protruding axonal and dendritic processes that weave through varying depths. The ability to image various depths is an invaluable attribute of LCM.

The administration of fluorescent chemical calcium indicators (CI; i.e., molecules that bind to Ca²⁺) in organisms, changes spectral properties when exposed to Ca²⁺. In this research study, the calcium binding protein (BAPTA) is administered to the specimen and chemically binds to Ca²⁺. The CI changes conformation (i.e., shape), when BAPTA binds with Ca²⁺ and undergoes laser stimulation. This group of actions results in a quantifiable change in fluorescent brightness (ΔF) when stimulated by 488 nm laser during an action potential. After the refractory period Ca²⁺ quickly dissociates from BAPTA, allowing the fluorescence to return to baseline.

Ca²⁺ increases inside neurons when they fire an action potential. If the Ca²⁺ ion is bound to a fluorescent calcium indicator, an increase in fluorescence can be observed via LCM. Ca²⁺ increases inside of a neuron during an action potential when Ca²⁺ is ingested at the axon terminals to facilitate neurotransmitter release. LCM and CI are invaluable tools in neuroscience and therefore, the instrumentation used in this study.

The encoding of sensory information and eventual conversion of this information into motor commands, although well documented, holds much to discover. It is known that incoming information is biologically encoded in the form of action potentials across neuron populations, but neuroscientists have yet to reveal how this information is recoded into appropriate motor output. Reticulospinal neurons in the larval zebrafish mediate
sensorimotor transformations. Understanding the interrelated functionality of zebrafish reticulospinal neurons and 5-HT may lead to a greater understanding of the sensorimotor transformations that take place in fish and humans alike. CI is one part of a larger movement to understand exactly how.

**Specific Hypothesis**

The goal of this research is to determine whether 5-HT directly modulates the Mauthner neurons in larval zebrafish. The basic method involves bath application of the exogenous selective serotonin reuptake inhibitor (SSRI) fluoxetine, more commonly know as Prozac. Stimuli will be provided via an electronically controlled “tapper” that will evoke an auditory startle response via cranial nerve VIII innervations. The Mauthner neurons were selected for experimentation primarily because of their large size and distinctive morphology. Mauthner neurons are easily located and identified, and thus can be consistently and reliably recorded. Moreover, there is a growing body of research on these particular neurons that strongly suggests the likelihood that 5-HT directly synapses onto Mauthner neuron dendrites (McLean & Fetcho, 2004b). This suggests that the modulation of spinal circuitry may occur pre-synaptically, at the reticulospinal neurons themselves. I postulate that serotonergic axons synapse directly upon the dendrites of the entire M-series reticulospinal group. To test this theory, I put forth two specific hypotheses pertaining to this empirical research study.
Hypothesis 1. Sensory-evoked Mauthner Ca$^{2+}$ responses correlated with the initiation of movement will significantly increase in magnitude from the administration of fluoxetine which is expected to increase 5-HT neurotransmission in the brain.

Hypothesis 2. Sensory evoked Mauthner Ca$^{2+}$ responses correlated with the initiation of movement will increase in latency (i.e., timing) in the presence of fluoxetine.
Method

Subjects

The subjects were six zebrafish (Danio rerio) larvae, approximately 4-8 days post fertilization. A Mauthner neuron was selected in each fish for experimentation. The larvae were brought down to room temperature, (~22°C) before experimentation. All animal subjects were raised and handled according to Humboldt State University institution animal care and use committee standards (IACUC08/09.P.45.A).

Materials

Imaging in this experiment was obtained through the Olympus FluoView 1000 laser scanning confocal microscope. Stimulus were provided via a specially designed electronic “tapper” which consists of a micropipette attached to a piezoelectric crystal that is controlled via microscope software. The tapper makes contact with a slide coverslip in which the specimen is agar fixed and inverted upon a specially designed well. This well has been constructed to bathe the specimen in either egg water or drug solution of choice. Fluid is easily injected or extracted with ease and accuracy, which allows for a repeated measures experimental approach consisting of pre treatment, treatment, and washout trials respectively. This device consists of a central well fed by a small plastic tube connected to a 10ml syringe containing the solution of choice. By retracting or depressing the syringe plunger, fluid levels can be lowered to the point of extraction or raised to the point of bathing the specimen.

Subjects underwent injections of 75% solution calcium indicator dye, Oregon-green dextran bis-(o-aminophenoxy) tetraacetic acid (BAPTA, 10,000 molecular
weight, Sigma). Zebrafish will be anesthetized with tricaine, 0.02% 3– amino benzoic acid ethyl ester (MS222, 261.29 molecular weight, Sigma). To prevent behavioral responses to the stimuli, zebrafish larvae were administered a 50% nicotinic acetylcholine receptor antagonist (nAChR), 10µM solution, 14-acetylvirescenine (Curare, 465.58 molecular weight, Sigma), which blocks neuromuscular transmission and thus will prevent severe behavioral movement artifacts via temporary paralysis. The main treatment drug in this study is an (SSRI), 10µM solution of -N-Methyl-γ-4 (trifluoromethyl) phenoxybenzenepropanamine hydrochloride (Fluoxetine, 345.79 molecular weight, Sigma).

**Design**

The experimental design of this study utilizes two separate repeated measures analysis of variance (ANOVA) tests with three between group factors representing treatment type (e.g., egg water, drug treatment, egg water wash out). Two separate analyses were run to determine fluorescence increases over pre-stimulus baseline (ΔF) and changes in latency (ΔT). This type of design is beneficial in terms of statistical power and incorporation of the variable of repeated measurement.

**Procedure**

The subjects were first injected with calcium indicating dye via glass pipette into the caudal spinal cord using a digital micro-manipulator and air pressure injector. These injections will be performed by Ethan Gahtan, Ph.D. They were then be placed into an
 incubator (approximately 27°C, 14h/10h light/dark cycle) overnight to allow for recovery and diffusion of the fluorescent dye throughout the descending axonal system. The following day, the larvae will were anesthetized with Tricaine. Immediately following, subjects were administered Curare. This chemical blocks the propagation of action potentials at the neuromuscular junction which induces paralysis while permitting functional nervous system activity.

For image acquisition, larvae were then be placed upon a microscope cover slip and immobilized in a 1.2% agar solution. The slip was then inverted and adhered to the top off a specially designed well that allows for the adjustment of fluid levels (see Figure 6).

*Figure 6. Diagram of custom designed experimental well. This specially designed apparatus allows for the rapid bath application of solutions.*
The above procedure allows for pre drug trials, followed by the treatment, followed by a washout trial. In this repeated measures type of experiment, the potential timing of onset along with that of diminishing effects can be observed and measured. After the specimen was secured in agar and is ready for experimentation an initial scan is performed to confirm the presence of backfilled reticulospinal neurons. Ca²⁺ readings are achieved via 488nm laser penetration of the specimen hindbrain. Spinal injections of this nature are not always successful, and spinal axons will sometimes fail to adequately ingest the necessary amounts of dye for viable experimentation. This process is further complicated by opaque pigmentation in the zebrafish skin that often obstructs imaging. After confirmation of the fluorescent dye in Mauthner neurons was established, an anatomical snapshot was taken for future reference regarding position and orientation of the cell in question. Upon completion of the anatomical scan, the Mauthner neuron is magnified and centered for analysis.

Experimentation began with the researcher drawing a line scan, through the center of s Mauthner neuron cell body. A line scan in LCM refers to a region of interest (ROI) being represented as a single line drawn across the cell body. Inside the confines of the drawn line, the computer averages the pixilation values as they change. ∆F levels will be recorded only in the area of this particular line. The main advantage in this type of scan lies in the speed of the image acquisition. A scan of the entire cell is relatively slow and computationally costly than a line scan. Furthermore, this method exposes the specimen to a greater amount of laser light stimulation. An abundance of laser stimulation could result in cell damage or the firing of an unnecessary action potentials Keep in mind that
reticulospinal neurons operate essentially as rhythmic pattern and escape circuitry controllers. Unnecessary activation may reverberate throughout the reticulospinal network causing behavioral artifacts. Even a slight perturbation in zebrafish body movement could cause the cell under scrutiny to move from the designated ROI, thus challenging the integrity and accuracy of the data being acquired.

After a scan line has been correctly drawn through the cell body and the time controller has been instructed as to the timing parameters, the experiment begins. Approximately 2000 scans will occur in approximately two seconds, at the rate of 1 scan per millisecond. At approximately 400-750 msec into the scanning the electronic tap is delivered upon a nearby area of the cover slip. This is provided on queue by the microscope itself, insuring that its delivery will be precise and in synchronization with the rest of the procedure. Through a triggering option, the microscope upon command produces a 5V transistor-transistor logic (ttl) pulse. This electrical pulse is inputted via Bayonet Neill-Concelman (BNC) cable into an A-M Systems Isolated Pulse Stimulator. This device produces a prescribed electrical output that is used to activate the piezoelectric crystal and produce the distinct tap. Stimulus intensity (i.e., voltage, duration, and frequency) are manually adjusted on the stimulator control panel, which can also accommodate varying strengths or frequencies.

In the case of this experiment, the output voltage is set at 10V for 2ms. Although the stimulator output was a pre-programmed voltage, the exact amount vestibular activation will vary from fish to fish. This disparity is due to several factors, such as fish orientation, health, and neurological maturation. Therefore, stimulus intensity will be
set at approximately 5% above the threshold that elicits an observable behavioral response (i.e., muscle twitch as observed through the microscope). This “tapper” method is a time tested method of producing an action potential in RS neurons.

Upon activation of a trial, the stimulus is delivered and any change in the magnitude of fluorescence $\Delta F$ is recorded. Immediately following the two second scan is a two minutes interval of rest. This pause in activity is serves to restore the specimen to a homeostatic state. Once homeostasis has been achieved the nervous system has returned to a pre-stimulus condition and is ready to again process stimuli. This time lapse reduces the organisms’ chances of becoming behaviorally conditioned to stimulus. This cycle of 2sec scans followed by two minutes of time is repeated five more times. This block of data is followed by 20 minutes of down time before being initiated again. This cycle occurs a total of three times for grand total of 15 scans taken over the course of 90 minutes. This data block will represent one treatment condition, of which there are three. At the end of the first treatment condition (i.e., egg water), the drug fluoxetine is administered and the process begins again. Following the end of this second treatment condition, the Fluoxetine is extracted and replaced back with egg water and the process is repeated for a third and final time. In total, a full round of three treatment conditions comprises 45 individual line scans and takes 4.5 hours to complete.

It is important to note that because the scan begins with laser excitation at approximately 300msec, a concern exists that the laser excitation itself could serve as conditioned stimuli, thus evoking a response that could be misinterpreted in terms of response timing. The possible undesired effects of laser conditioning are addressed
through the observance of Ca²⁺ responses during baseline trials. If a conditioning effect is observed (i.e., a Ca²⁺ response occurs in response to laser excitation), the stimulus is adjusted to take place at varying times during the scanning process (e.g., 100ms, 400ms, and 600ms).

It is also important to note that in the larval stage of zebrafish development, drugs are absorbed through the skin. Since the transdermal rate of absorption in larval fish is unknown, the timing of drug administration can be later compared to the strength of the observed response, thus possibly alluding to zebrafish pharmacodynamics. At the end of a full experimental session, the specimen is administered a lethal dose (0.1% solution) of Tricaine.

A scan that exhibits a change in ∆F greater than 10% from baseline is considered responsive and analyzed accordingly. This is further confirmed visually by the characteristically well defined Ca²⁺ response which consist of a sharp rise (i.e., leading edge) and slow decay, reminiscent of a saw tooth form (see Figure 7). Response rate for each condition is analyzed in this manner and is represented as a percentage.

Quantifying ∆F for statistical comparison is much more complex. First, every line of data leading up to the stimulus onset is averaged. This number represents a stable baseline by which the degree of change can be effectively established. The task is performed to counteract any differences regarding fluorescence values. Inevitably, some cells may idle more erratically than others or contain varying amounts of the dye. After establishing the baseline, a sliding average of every ten scans is then performed.
Figure 7. Stereotypical Ca²⁺ response. After the delivery of the stimulus (shown as faint white line to the left of the spike) a typical Ca²⁺ will show a rapid rise in fluorescence followed by a quick return to baseline, making a characteristic saw tooth.

A technique utilized in “smoothing” data of a noisy nature, such as Ca²⁺ imaging, is then performed. Afterwards, a normalized to baseline method is employed in which each line of the smoothed data is divided by the previously established baseline average. These data are then inputted into an Excel formula array that establishes the maximum ∆F achieved within a 750ms timeframe following the stimulus. This value, the peak amplitude of the Ca²⁺ response, represents the total percentage of ∆F increase evoked by the stimulus.

To determine response latency (i.e., response time) the time marker in which the response is first observed is recorded and subtracted from the stimulus delivery time. This proves at times to be a difficult endeavor, in that the cells under observation serve to produce movement which in turn can distort and mask the exact moment of cellular activation. The problem is overcome by recording the moment of response regardless of a
distinct leading edge, which at times will appear as a downward spike as the cell
temporarily moves from focus. Regardless of the presence of a distinct leading edge, this
method is an acceptable means of gauging the response latency with a high degree of
accuracy.

In both the cases of ΔF and latency values, an overall mean for every five scans is
generated for statistical analysis. For an entire research session consisting of three
treatment conditions totaling 45 scans, nine values will come to represent ΔF and latency
data. These values will be compared statistically in repeated measures ANOVA, which is
suitable for this type of research design. A separate repeated measure ANOVA will be
employed for both the ΔF and latency data.
Results

Results indicate no significant difference in Ca²⁺ fluorescence $F(2,28) = 1.74 \ p = .21$, partial $\eta^2 = .11$. A slight increase was observed in Ca²⁺ levels over the course of the experiments, with the pre-treatment base line having the lowest mean ΔF ($M = 21.2, SD = 6.9$), the fluoxetine trials having an intermediate value ($M = 23.7, SD = 7.8$), and the post treatment return to baseline having the highest mean ΔF ($M = 25.9, SD = 5$). This gradual increase in fluorescence represents a trend but was not statistically significant (see Figure 8).

*Figure 8.* Results for delta F. Results show an increasing mean ΔF throughout the treatment conditions, (Pre Fluoxetine, Fluoxetine, and Washout). The bars in this graph represent the average percentage of fluorescence for each treatment condition. Also shown are error bars representing the standard error of the mean.
Results indicate no significant difference in latency, $F(2,28) = 1.45 \ p = .25$, partial $\eta^2 = .094$, although a gradual decrease in latency was observed, with the pre-treatment base line having the highest mean $\Delta F (M = 24.6, SD = 12)$, the fluoxetine trials having an intermediate value ($M = 21.7, SD = 7.9$), and the post treatment return to baseline having the lowest mean $\Delta F (M = 20.7, SD = 7.4)$. These data show a decreasing trend in response time throughout the course of the experiment but the trend was not statistically significant (see Figure 9).

*Figure 9.* Results for response latency. Results show a slightly decreasing mean $\Delta T$ throughout the treatment conditions, (Pre Fluoxetine, Fluoxetine, and Washout). The bars in this graph represent the time of response in milliseconds. Also depicted are error bars representing the standard error of the mean.
Mauthner responsiveness (i.e., firing or no firing) was also assessed and showed no significant fluoxetine treatment effects. The response rate for the pre treatment group was 79%, the Fluoxetine group was 87%, and the post treatment group was 79%.

**Supplemental Control Results**

The Huynh-Feldt adjustment was used to correct problems with sphericity. Control group results indicate no significant difference in Ca²⁺fluorescence $F(1.95,4) = 1.62, p = .31$, partial $\eta^2 = .447$. Mean values show an increase in fluorescence throughout the experiment, block 1 ($M = 47.5, SD = 14.3$), to block 2 ($M = 46.3, SD = 9.8$), and block 3 ($M = 62.3, SD = 8.4$).

The Huynh-Feldt adjustment was used to correct problems with sphericity. Control group results also indicate no significant difference in latency $F(1.95,4) = .844, p = .49$, partial $\eta^2 = .297$. Mean values show a decrease in response time throughout the course of the experiment, condition 1 ($M = 33, SD = 2.5$), condition 2 ($M = 32.6, SD = 5.8$), and condition 3 ($M = 28.9, SD = 2.4$).
Discussion

The main hypothesis, that tap-evoked Mauthner cell activity in larval zebrafish, as measured through fluorescence calcium imaging, would be modified by bath application of fluoxetine, was not supported. This hypothesis was based on previous research showing that serotonin influences motor behavior in the zebrafish larvae, and that the dendrites of the Mauthner neuron and other descending neurons in the hindbrain are colocalized with serotonin immunoreactivity (McClean & Fetcho, 2004). A slight increase in Mauthner cell activity was observed throughout the course of the experiment (45 trials over approximately 4 hours). This could be attributed to Ca²⁺ dynamics. With each action potential and the subsequent ingestion of Ca²⁺ at the axon terminals, it is possible that an ambient amount of the Ca²⁺ CI may have become disassociated inside the cell. In this scenario, a gradual buildup may account for a slight increase in ∆F over time.

No significant difference was found in latency of Mauthner responses. However, a gradual decrease in latency was observed throughout the course of the experiment, paralleling the gradual increase of ∆F that was observed. This effect may be due to factors outside of the influence of 5-HT. It appears that what was observed in the latency data was a decrease in reaction time most likely attributable to the well documented phenomena of neural sensitization. The control data shows both the phenomena of sensitization and increase in ∆F, indicating that these effects were a natural occurrence.

An interesting finding of this study was the comparison of the probability that the cells would or would not react to the stimulus (i.e., they would fail to fire), as is sometimes the case regardless of scan block or treatment. Surprisingly, both the pre and
post treatment conditions displayed the exact same response rate of 79%, while the Fluoxetine treatment condition responded 87% of the time. It is important to note that the 79% responsiveness for the pre and post treatment groups was identical. This constitutes an increase of 8% in responsiveness in the presence of 5-HT. This finding could simply be an anomaly or coincidence, or, perhaps an unanticipated glimpse into an effect of 5-HT on Mauthner function that is purely speculative.

**Limitations**

Rigorous scientific experiments of this caliber are often riddled with unanticipated difficulty and this study was no exception. Given the exploratory nature of this research along with the sophistication of instrumentation, many setbacks and/or adjustments to the experimental procedure were made along the way. Every step of this study was labor intensive, time consuming, and complex. Nonetheless, these data have shed some light upon the influence of the 5-HT on the Mauthner neuron.

The first limitation to this study worth mention is the problem regarding statistical power because of the small sample size. Biological research studies are often forced to cope with a less than desirable sample size primarily because of the time consuming and labor intensive manner in which specimens are prepared and results are analyzed. Specimens must endure a multitude of preparatory steps simply to pass as suitable for experimentation and often display varying degrees of reactivity. Slight variations of health and development all also alter experimental consistency. Although this study has a small sample size, a total 315 individual scans were performed requiring 32+ hours of
laboratory equipment usage. Scan conversion to quantifiable means was equally laborious, and easily exceeded 50 hours to convert the raw data to a quantifiable state. These are some of the reasons why despite the strong desire for a larger sample size, it is a task easier said than done.

A noteworthy limitation of the research involves the stimulus type (i.e., the tapper) and manner in which it was used. The orientation, intensity, and distance of the stimulus were difficult to control for. Disparities in stimulus delivery could include but are not limited to, distance, angle of incidence, and intensity. In perfect world, the delivered stimulus would be unwaveringly and dynamically consistent in every single trial and subject. However, given the confining nature of the microscopic work area in which the experimental procedures have taken place, this can be a daunting task. Perhaps a better means of providing a stimulus involves the use of a strategically and permanently placed sound device, or, even the incorporation of light stimulation through the use of a light emitting diode that would provide the exact strength and distance from the specimen. The Mauthner neurons do in fact receive optical inputs, so this strategy may be one worth consideration.

Other possible limitation revolve surround the use of the drug fluoxetine. Despite being an established SSRI, which is expected to increase 5-HT neurotransmission, fluoxetine is also a 5-HT\textsubscript{2c} antagonist (Chong, Holmqvist, & Drapeau, 2003). Previous antibody research indicates the presence of 5-HT associated immuno-reactivity in the vicinity of the lateral and ventral Mauthner dendritic fields, but no information indicates which receptor sub-type or combination thereof is present (Mclean & Fetcho, 2004b). It
is possible that the 5-HT$_{2C}$ receptor is necessary for 5-HT activity in Mauthner neurons, and that its fluoxetine induced antagonism is responsible for the lack of a significant difference in $\Delta F$.

When selecting a treatment drug that alters 5-HT activity, a researcher must choose from a variety of agonists and antagonists that typically operate upon only a few receptor sub types. The use of an established drug like fluoxetine was chosen in this study with the idea in mind that through reuptake inhibition we could effectively promote the transmission of 5-HT across all receptor sub types.

Another possible lack of significant results may be in the bath application method used in this study. Reticulospinal neurons receive input from several sources (e.g., lateral line, optic tectum, VII nerve; Sillar, 2009). Saturation of the entire zebrafish nervous system may inhibit afferent connections from the various sensory systems pathways. In this sense, exogenous bath application of 5-HT may inhibit neuronal activity upstream of the Mauthner neurons.

The largest possible limitation in which I have identified involves the overall experimental methodology. Previous research involving zebrafish, lamprey, and *Xenopus* all show, in various ways, an alteration of swimming behaviors when subjected to 5-HT (Batueva, 2002; McDearmid, Scrymgeour-Wedderburn, & Sillar, 1997; Soffie et al., 2009). As we know, these swim episodes are directly controlled by reticulospinal neurons, and the activity of these neurons should directly coincide with locomotor behaviors (e.g., fictive swimming; Soffie et al., 2009). The problem herein lies in the choice of measurement used to analyze reticulospinal cellular activity. The above
referenced research studies indicate changes in the frequency, intensity, and duration of swimming behaviors. Changes in ΔF may not be the most appropriate of means to test the type of change we are likely to find, given that recorded CI traces represent a single action potential rather than a series. A more appropriate means of assessment may simply be a micro-electrode probe placed in the Mauthner soma. In this method, a serious of taps, or the use of glutamate could be used in eliciting a series of activity bursts recoded in form of voltage spikes. It would be simple to asses if at the Mauthner neuron itself, not the subsequent behavioral movements, decreases or increases in firing frequency, whether by increasing burst width or downtime in this manner.

**Directions for Future Research**

Along with changes discussed above, future research may wish to explore the use of different 5-HT influencing drugs (e.g., buspirone, ketansarin, or quipazine). Along with the use of varying 5-HT drugs, a future researcher may wish to utilize a parametric protocol. Our study uses an established 10μM concentration of Fluoxetine, but it may be beneficial to step up and down the concentrations in increments of 5μM to address the possibility of over or under saturation.

It may also be beneficial to future research to test zebrafish larvae of varying stages of development (e.g., 4dpf, 8dpf, 10dpf). Previous research suggests that 5-HT alters zebrafish behavior in different opposing ways depending upon age (Brustein et al., 2003). Perhaps along with implementation of parametric design, the within group variable of age could be incorporated as well.
Conclusion

Despite the failure to reject the null, visually, 5-HT appears to affect Mauthner activity. Scans in the fluoxetine treatment group often appear distorted and noisy. Ca^{2+} scans in some cases exceeded the requirement 10% ΔF threshold indicative of a response in the absence of a discernable Ca^{2+} trace. Ambient noise spikes both preceding and after the influence of the stimulus often appears unstable. I can only speculate as to why this phenomena is observed, but believe the answer may lie in the possibility of 5-HT being influencing the activity of glycine releasing neurons that act as noise filters and synapse upon Mauthner neurons (McDearmid, Scrymgeour-Wedderburn, & Sillar, 1997). An interactive effect regarding other neurotransmitters and their possible influence is beyond the scope and necessity of this study. Yet nonetheless, is something to take into account when contemplating the possible intricacies regarding the influence of 5-HT as a small part of a possibly much larger and complex picture.

In conclusion, I believe this study has shown the effects of CI over repeated exposure, and the process of neuron sensitization. If anything, this study has shown the need for a larger sample size, the implementation of a parametric protocol, or re-evaluation of the methodology used in establishing the modulation of Mauthner neurons by serotonin. Mclean and Fetcho (2004b) provide compelling visual evidence for Serotonergic input into the Mauthner neuron through immunolabelling. When faced with their micrographs which clearly indicate antibody labeling in a consistent and clustered proximity to Mauthner dendrites and its homologs, I find it difficult to believe that 5-HT
does not play some type of measurable role in the modulation of activity in these neurons. Regardless of the findings and shortcomings of this study, I am confident that 5-HT does directly influence Mauthner activity, and believe that perhaps one day it will be quantifiably demonstrated and fully understood.
References


0022-3077/08


subtypes in the neonatal mouse spinal cord. *Journal of Neurophysiology, 92*, 1566-1576. doi:10.1152/jn.01181.2003


