EXPLORING THE SOCIAL BEHAVIOR NETWORK IN THE DEVELOPING ZEBRAFISH BRAIN

by

ALLISON HOSLETT

A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2019

An Abstract of the Thesis of

Allison Hoslett for the degree of Bachelor of Science
in the Department of Biology to be taken June 2019

Title: Exploring the Social Behavior Network in the Developing Zebrafish Brain

Approved: _____

Philip Washbourne

Research for neurodevelopmental disorders characterized by social deficits, such as Autism Spectrum Disorder, has helped increase the quality of life in individuals and families afflicted by these diagnoses. This research aims to further understand the neuronal network underlying social behavior in the developing brains of zebrafish (*Danio rerio*), which is currently poorly understood. The zebrafish is an experimental animal model that shares relevant cellular pathways and social behaviors that are conserved between vertebrate animals. We use genetic and behavioral research techniques to identify populations of neurons that are necessary to recognize biological motion, a critical component of typical social behavior. The genetic tools we use allow for cell tracking and nitroreductase-mediated cell death. After the targeted ablation of neurons in different areas of the brain, we use a behavioral assay to measure the social indices of individual zebrafish larvae. This assay projects dots that imitate the presence and movement of another fish while tracking the fish's interactions with the dots in real-time. This is then calculated into a social index. We were able to identify neuronal populations that, after ablation, severely reduce typical social behavior. Using and improving this approach will allow us to identify a more complete picture of how the social circuit functions and which neuronal populations are involved. Unraveling the social circuit has the potential to increase early identification and targeted treatments of patients with neurodevelopmental disorders that are characterized by impairments in typical social behaviors.

Acknowledgements

I would like to thank Professor Philip Washbourne and Senior Research Associate Alexandra Tallafuss, for giving me the opportunity to work on this project with them and critically evaluate aspects of research in neuroscience and molecular biology. I am with the upmost gratitude to have had these two individuals and the rest of the Washbourne Lab give me a valuable, challenging, and rewarding experience. I would also like to thank Tim Mason and Adam Christensen for taking time to provide me the tools I needed in the University of Oregon zebrafish facility. Another acknowledgement the Clark Honors College Representative on my thesis committee, Casey Shoop. As a professor, he upholds the ideals of the CHC. He always challenges his students and encourages them to reach their true potential in the critical analysis of literature. He is one of the professors who I believe genuinely shaped my CHC experience. Finally, I would like to thank my parents, Steve and Julie Hoslett, who made all of my memorable and life-altering experiences with the Clark Honors College at the University of Oregon possible. Without their support, I would not have been able to fully explore and reach the potential in my academics and research.

Table of Contents

List of Figures

Introduction

Abnormal Neurodevelopment in Autism Spectrum Disorder

Research in neurodevelopmental disorders, particularly for autism spectrum disorder (ASD), has helped increase the quality of life in individuals and families afflicted by these diagnoses. ASD is associated with abnormal early development in the brain leading to altered neural connectivity compared to a typically developed brain. It is characterized in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5)[1] by deficits in social communication and interaction, and by restricted, repetitive patterns of behavior, interests, or activities. Current research has found that children diagnosed with ASD usually have neural activity patterns of local over-connectivity in the frontal and occipital brain cortices, and an under-connectivity of global circuitry between all brain cortices (Lord *et al*., 2018).

During neurodevelopment, a forebrain subdivision called the telencephalon develops into the cerebral cortices and the basal ganglia. The neural activity within and between these regions of the brain is involved in visual-motor processing, responsible for using visual information to coordinate motor responses. The abnormal motor learning patterns related to social deficits in children with ASD are suggested to result from implicated neural circuitry involved in visual input and motor output integration (Nebel *et al*., 2016).

[1] The DSM-5 is the most recent version of the diagnostic tool published in 2013 by the American Psychiatric Association (APA).

Disrupted development of neural functions in ASD is associated with accelerated brain growth during early life stages. Research shows trends of larger than average brain volumes in children with ASD compared to those with typically developed brains. During later life stages, these larger brain volumes generally normalize to meet the average adult brain size (Courchesne *et al.*, 2011). An important aspect of autism research the networks of neurons implicated during abnormal development processes and their involvement in producing social communicative and behavioral deficits.

The Zebrafish Model (*Danio Rerio*)

Zebrafish *Danio rerio* are important vertebrate animal model in neuroscience for developmental and genetic research purposes. Zebrafish have relatively simple nervous systems and the species has undergone evolutionary pressures for the rapid development of their functional sensory systems and innate social behaviors. In addition, their transparency in early life stages allows for easy application of optogenetics[2], a useful tool in research for tracking molecular mechanisms of single cells or the activity of larger circuits of cells (Sumbre & de Polavieja, 2014).

Evolutionarily conserved biological pathways and social behaviors in humans and vertebrate animal models make zebrafish relevant experimental models for studying the complex pathology of neurodevelopmental disorders. There is a rapidly increasing number of available zebrafish mutants that act as experimental models for human neurodevelopmental, neurological, and neurodegenerative disorders and diseases.

[2] Optogenetics is a biological technique that uses light-sensitive, fluorescent proteins to control and monitor cellular and subcellular processes in living tissues.

Among these are models of ASD, Parkinson's disease, and Alzheimer's disease (Sumbre & de Polavieja, 2014).

Neuroanatomical regions of the zebrafish telencephalon (forebrain) have been proposed to be functionally homologous to the mammalian telencephalon, the region of the brain that develops into cerebral cortices and subpallial structures responsible for regulating social behaviors through the sensory relay systems of the hindbrain (Mueller, 2012; Stednnitz *et al*., 2018). In molecular developmental genetics, functional homologies refer to the conserved roles of genes regardless of the presence of structural homology (Love, 2007).

Zebrafish are also a powerful genetic model for studying neurodevelopment because of their transparency during early life stages (Meshalkina *et al*., 2016). The clarity of the zebrafish embryo and larvae allow for real-time tracking of neuronal populations using fluorescent proteins, such as GFP (green fluorescent protein) and mCherry (red fluorescent protein, RFP). Fluorescent protein expressions are genetically controlled in transgenic zebrafish through the implementation of the bacterial Gal4/UAS system, a binary expression system that can be utilized in tagging cell populations of interest and autonomously[3] targeting them for ablation (death) (Halpern *et al*., 2008).

[3] Cell autonomy occurs when only genotypically mutant cells exhibit a mutant phenotype; neighboring cells are unaffected if they do not have the genetic trait.

Forebrain Involvement in Social Behavior

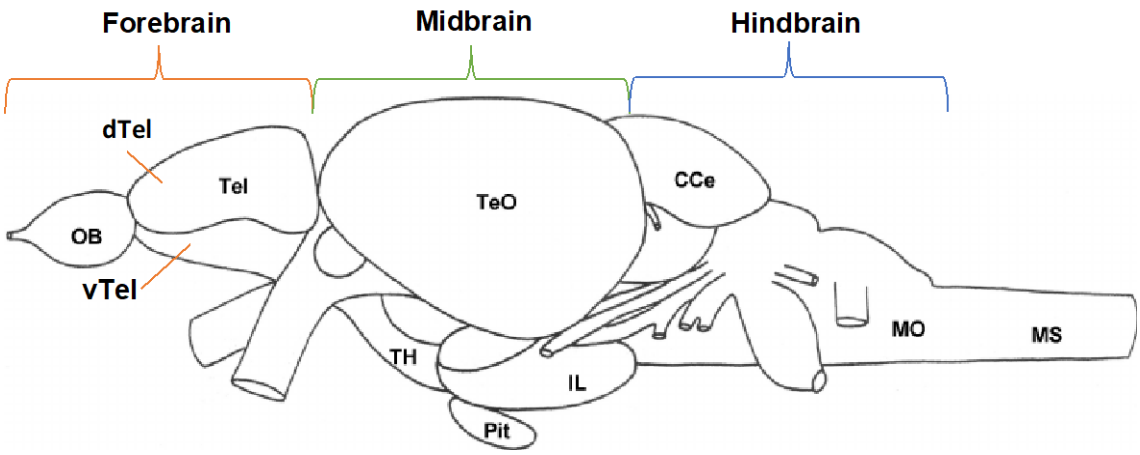

Figure 1: Sagittal View of the Zebrafish Brain.

Forebrain: olfactory bulb (OB), dorsal telencephalon (dTel), ventral telencephalon (vTel); Midbrain: optic tectum (TeO), tuberal hypothalamus (TH), pituitary (Pit), inferior lobe of hypothalamus (IL); Hindbrain: corpus cerebelli (CCe), medulla oblongata (MO), medulla spinalis (MS) (Wullimann *et al.*, 1996).

Recent research on the neural circuitry of social behavior in zebrafish discovered a genetically targetable group of neurons in the ventral forebrain, particularly the ventral telencephalon (vTel, Figure 1), that is involved in social orienting behavior (Stednitz *et al.*, 2018). The research utilized cell-specific Gal4 driver lines to inactivate the population of neurons and discovered consequential impairments in social orienting behavior, which refers to the ability to visually process aspects of form and motion in neighboring zebrafish during social interaction and respond with appropriate orientation of the body. These recent findings have raised questions about

the role of the brain's social behavior network and the development of neural processes that use visual social cues to guide social behaviors (de Polavieja & Orger, 2018).

The group of neurons identified to be necessary for social orienting behavior were expressed in the medial areas of the ventral forebrain using the *y321*:Gal4 driver line (Figure 2). Social behavior of the zebrafish was measured based on their degree of body orientation. Zebrafish showed significant impairments in normal behavior after the chemo-genetic ablation[4] of *y321* neurons compared to a control group. Using the same tests, researchers found that a different group of neurons expressed by the *dlx*:Gal4 driver line in lateral regions of the ventral forebrain were not found to be necessary for preserving typical social orienting behavior (Stednitz *et al.*, 2018).

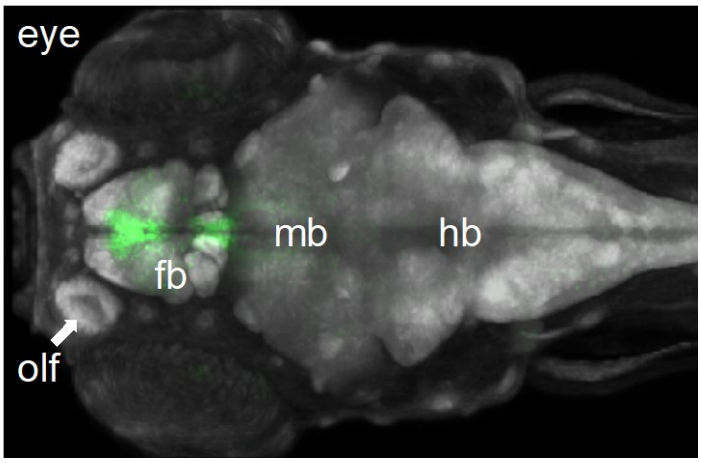

Figure 2: Expression pattern of *y321*:Gal4 zebrafish at 7 dpf.

The *y321*:Gal4 driver line strongly expresses GFP in regions of the ventral forebrain.

[4] Chemo-genetic ablation uses a pharmacological agent to induce autonomous cell death in cells expressing a toxin gene.

The cell autonomous methods of cell suicide used by Stednitz and colleagues (2018) to elucidate neuronal populations involved in the zebrafish social behavior network provides a framework for this research project. In particular, our research focuses on using these methods in zebrafish larvae versus adults to help deduce neuronal populations essential for social behavior development. In preliminary rounds of testing, chemo-genetic ablation of the *y321* population of neurons in larvae (age 18 dpf, days past fertilization) showed social behavior deficits. These positive results provided motivation to begin exploring the use of genetic and behavioral tools with other transgenic lines that differ in their cell-specific expression patterns, incorporating the study of neuron populations.

Biological Motion as a Measure of Social Behavior

The relationship between genetics and biological motion processing has become an increasing point of interest in research related to ASD. Cognitive processing of biological motion refers to the ability to extract visual information from the movements of others and use it to guide subsequent behavioral responses. Researchers Annaz and colleagues (2012) found that children diagnosed with autism did not significantly attend more to a point light display image representing a walking person than another one with scrambled dots (Figure 3A). Contrarily, children with typically developed brains did show a significant preference of cognitive attention focused on the walking man versus the scrambled dots (Figure 3B). Understanding how biological motion processing

affects social perception and collective behavior can increase the understanding of neural epigenetics[5] related to abnormal sensory-motor behaviors in ASD.

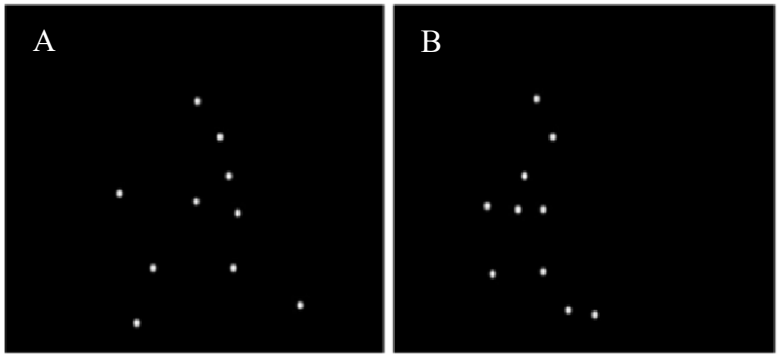

Figure 3: Point light displays of biological motion.

(A) Point light display representing the image of a walking person; (B) Point light display with random assortment of dots (Annaz et al., 2012).

Heritability of Biological Motion Processing

Impairment in the neural circuitry dedicated to the brain's ability to process biological motion has been linked to compromised social cognition in ASD. It is suggested that there is a genetic basis behind these differences in the neural processing of biological motion. Based on experiments of heritability in twin studies, Wang and colleagues (2018) suggest that children diagnosed with ASD may have a genetic predisposition for abnormal neural processing of visual cues of joint movements, known as local biological motion processing, during critical developmental periods in early life stages. Attending less to local biological processing may then result in less cognitive attention towards aspects of biological motion processing that are later learned, responsible for the processing of global configurations that perceive entire skeletal

[5] Epigenetics is the study of heritable changes in genetic phenotypes, or gene expression, that do not involve changes in the DNA sequence itself.

structures (Wang *et al*., 2018). The involvement of genetics in biological motion processing and its disruption in children with ASD makes it an appealing area of interest in studying the neuronal network of social behavior.

Social Cues of Biological Motion in Zebrafish Shoaling

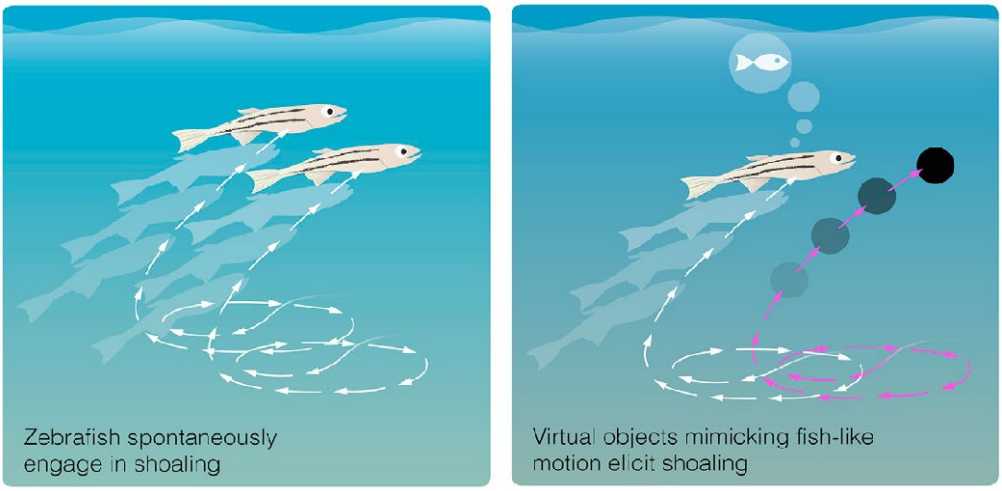

Figure 4: Biological Motion in Zebrafish Shoaling.

Zebrafish innately engage in shoaling as a collective behavior. They will also engage in this behavior with a virtual moving dot if it exhibits the similar size and movement of a conspecific fish (Larsch & Baier, 2018).

A strong example of a collective behavior is shoaling, a well-defined social behavior of zebrafish. Shoaling is an innate behavior that is known to develop in larval zebrafish as early as 14 days of age. It is known to be an essential adaptation for predator avoidance, foraging, and coping with stress. The social behavior is characterized by a strong, mutual attraction to conspecific fish and the maintenance of this affiliation at a preferred distance (Larsch & Baier, 2018).

Using a virtual behavior assay, researchers Larsch & Baier (2018) were able to deduce the core aspects of biological motion involved in zebrafish shoaling at 17 dpf.

Their results established that the visual stimulus of a two-dimensional black dot with similar size and motion of a neighboring fish was sufficient to elicit instinctual shoaling behavior in juvenile fish (Figure 4).

The experimental assay utilized in this project was developed from Larsch & Baier (2018), using the projection of a moving, two-dimensional black dot below shallow dishes (Figure 5). This simple representation of another fish is formulated to mimic the swim kinetics of conspecific fish and trigger innate shoaling behavior of the experimental fish. The movements of the fish are tracked via camera under infrared (IR) illumination using Bonsai software, a visual programming framework designed to acquire and process data streams online. Its application to the field of neuroscience has been a significant advancement for data collection of dynamic behavior assays. For zebrafish in particular, is has the ability to continuously track their positions and orientations in real-time (Lopes *et al.*, 2015).

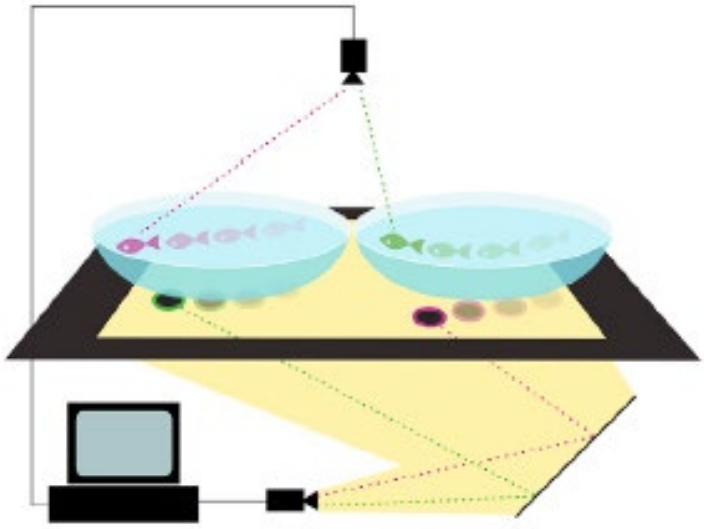

Figure 5: Virtual behavior assay.

The virtual behavior assay above illustrates the projection of moving black dots to individual dishes of fish that trigger shoaling. A computer with Bonsai programming is connected to a projector that facing a mirror that then projects the two-dimensional dots onto a screen underneath the individual dishes. A camera above the shallow dishes tracks the fish movements, with inputs into the Bonsai computer program. The location of the fish in their dishes throughout the experiment can be analyzed in accordance to the two-dimensional dot settings (Larsch & Baier, 2018).

A baseline for typical social behavior according to biological motion aspects showed differences in social index depending the size of the virtual dot (Figure 6; Larsch & Baier, 2018). The fish's level of affiliation increases as dot size increases until a maximum is reached, where it then begins to decline as the dot size continues to increase. This is possibly associated to the biological motion stimuli of a predatory threat by a larger fish. If no dot or a small dot is present, there is not enough stimulus to initiate shoaling behavior.

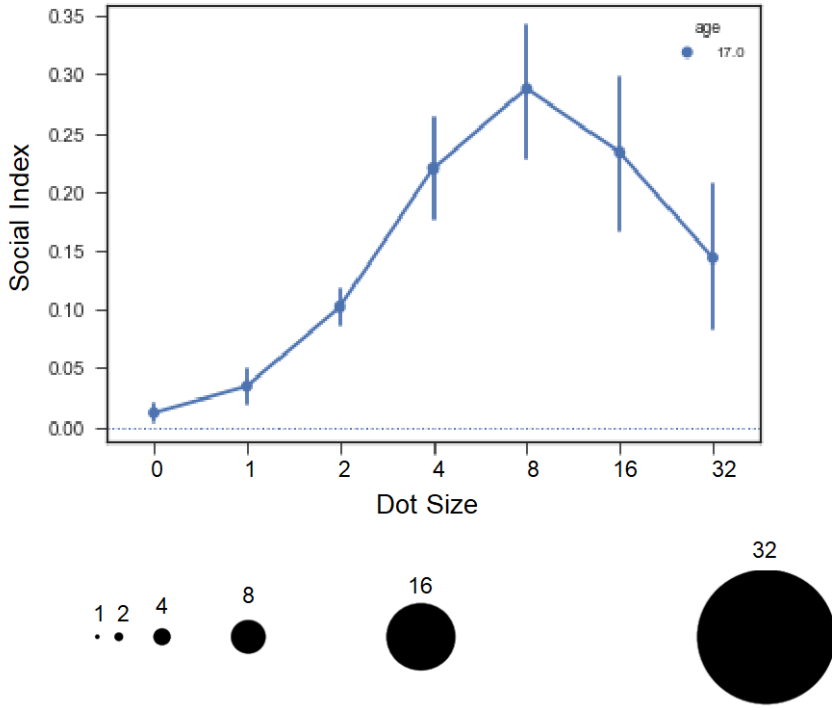

Figure 6: Social index versus dot size in zebrafish, 17 dpf.

Zebrafish have different levels of social affiliation with a projected dot depending on its size. Dot sizes are measured by diameter in millimeters (mm).

Research Overview

This project and future related projects are focused overall on the development of social behavior in the brain, and how abnormal neurodevelopment results in social behavioral deficits. The goal of this research is to make progress in the elucidation of the distinct neuronal populations involved in the zebrafish social behavior network. We use transgenic zebrafish with different expression patterns to measure the effects of neuron inactivity in areas of the brain such as the dorsal telencephalon (dTel, Figure 1) (Morita *et al.*, 1995). This work is meant to expand on the previously mentioned results with *y321* and *dlx* expression patterns that found a population of neurons in the vTel

that are necessary for social behavior. The overall importance of this research is its contribution to a deeper understanding of the implicated cellular pathways in abnormally developed brains that are responsible for social impairments.

Methods

NTR-Mediated Cell Ablation

Transgenic lines of zebrafish used in this project utilize the bacterial Gal4/UAS system for fluorescent labeling of cells and targeted cell ablation. The zebrafish are housed and maintained in the University of Oregon's Zebrafish Facility that supports biological research in the Institute of Neuroscience.

Gal4/UAS System

The Gal4/UAS system is incorporated into their genetic code and is under the control of a tissue specific promotor that determines what cells are targeted. The promotor will begin transcription of the Gal4 gene, producing small molecules that will bind to a short gene sequence called the upstream activated sequence (UAS). Activation of this site will drive expression of downstream genes based on their genetic purpose. One of them expresses a green fluorescent protein (GFP) and another expresses the bacterial *nfsB* gene and mcherry, a red fluorescent protein (RFP) (Figure 7). The *nfsB* gene encodes a protein called nitroreductase (NTR), an enzyme that catalyzes a reaction that then induces a chain of events for apoptosis (cell suicide).

In transgenic zebrafish with the *nfsB* gene, cell death is manipulated through use of a pharmacological agent, either metronidazole (MTZ) or nifurpirinol (NFP), acting as the NTR enzyme substrate. During treatment (directly in the zebrafish tank water), the cells expressing the *nfsb*-mcherry sequence will be ablated and neighboring cells will not be affected (Halpern *et al*., 2008; Bergemann *et al.*, 2018).

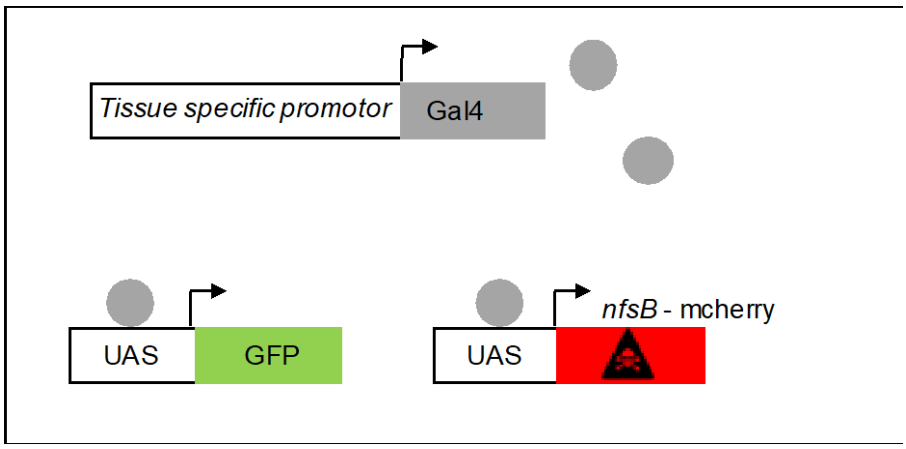

Figure 7: The bacterial Gal4/UAS system.

The tissue (cell) specific promotor will produce Gal4 protein molecules in the cells where the gene is expressed. The small Gal4 molecules bind to upstream activated sequences (UAS) of either GFP and/or *nfsB*-mcherry depending on the genetic code of the zebrafish. Expression of the *nfsB*-mcherry gene induces a chain of events resulting in cell death.

Metronidazole (MTZ)

The well-known drug that has been used for NTR-mediated cell ablation was the antibiotic metronidazole. The concentration (10mM) of metronidazole that was originally used in the first set of virtual behavior experiments was 10mM, as published in the Stednitz *et al*. article (2018) where it showed efficient neuron ablation in *y321* cells. However, the protocol stopped working and we began exploring new concentrations and conditions in order to produce a modified, working protocol. We tested two different manufacturers for the drug (Sigma Aldrich and ACROS), light versus dark conditions (aluminum foil covers), different concentrations (10mM, 12mM, and 15mM), and longer treatment times (18-24h). In addition, previous research had supported the use of DMSO (dimethyl sulfoxide) to help facilitate drug entrance into

cells (Mathias *et al.*, 2014), so we tested the addition of 0.1% DMSO to the original concentration of 10mM. The transgenic line of zebrafish used for these experiments were *mnx1*:Gal4, a gene which is expressed in motor neurons in the spinal cord. These cells are known to be easily accessible to pharmacological agents. Multiple rounds of *mnx1* fish at 3-5 dpf were treated with MTZ in Petri dishes.

Use of MTZ in juvenile zebrafish before virtual behavior experiments was either a 10mM solution in fish water overnight (17 dpf), or a 10mM solution in fish water for 24h (16 dpf) and 24h recovery period following the treatment.

Nifurpirinol (NFP)

NFP is a nitroaromatic antibiotic like MTZ. For this reason, its ability to be an efficient substrate in NTR-mediated cell apoptosis was assessed. Bergemann *et al.* (2018) found that the drug actually improved the reliability of NTR-mediated cell death compared to MTZ, even at a much lower concentration. In addition to its higher potency, the drug is also appealing because it is less toxic towards the zebrafish than MTZ.

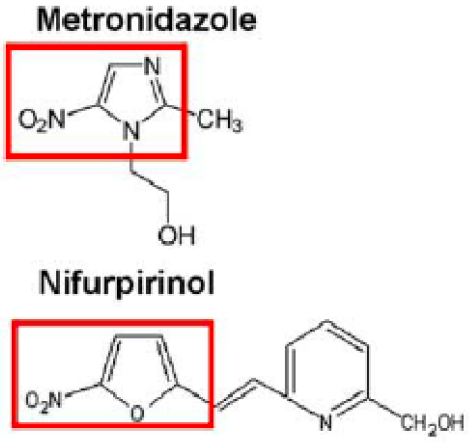

Figure 8: Molecular Structures of MTZ and NFP.

This figure indicates the nitroaromatic rings (red boxes) of metronidazole (MTZ) and nifurpirinol (NFP) (Bergemann *et al.*, 2018).

NFP was first tested in the *mnx*1:Gal4 zebrafish line at 3 dpf in Petri dishes to evaluate its cell ablation efficiency of *nfsB*-mcherry expressed (RFP+) motor neurons in the spinal cord. The RFP+ zebrafish were treated for 24h in a 2.5uM NFP solution with embryo medium (EM) made from a stock solution of 2.5mM NFP/DMSO. The control group was put in a Petri dish of EM only for the same amount of time.

Fluorescence Microscopy

Fluorescence microscopy is used as a tool in biological research to study cellular functions. It is attractive because fluorescent signals have high image contrast and specificity. Using fluorescence microscopy in optogenetics gives researchers the ability to study the structure and physiology of intact cells (Dobrucki & Kubitscheck, 2011).

We used transgenic lines of zebrafish containing UAS: GFP and mated them with zebrafish containing UAS: *nfsB*-mcherry (RFP). Embryos from the mating crosses were then collected into Petri dishes and 300-400 viable embryos were bleached and put into new Petri dishes (100 per dish). At 3-5 dpf zebrafish larvae are anesthetized using tricaine (MS-222) under IACUC[6] protocol and sorted using a Leica widefield fluorescence microscope into two new Petri dishes— (1) GFP+, RFP- and (2) GFP+, RFP+. Both groups are treated with MTZ/NFP in small tanks before being used for experiments in the virtual behavior assay.

Virtual Behavior Assay

After either MTZ or NFP treatment, zebrafish are transferred from Petri dishes to individual shallow dishes in the virtual behavior apparatus with a plastic pipette. There is fish water in and surrounding the dishes. Using the virtual behavior assay (Figure 5) we monitored 15 fish (18 dpf) for 3 hours, running two trials in a day to total 30 fish per experiment. Half of the total fish tested are GFP+ and RFP- (control group), and the other half are GFP+ and RFP+ (experimental group). Individual zebrafish movements and positions within the dishes are recorded and collected using an overhead camera. The information from the camera is processed in Bonsai using a python-based anaconda script.

[6] The Institutional Animal Care and Use Committee (IACUC) is responsible for overseeing research involving laboratory animals.

Confocal Microscopy

Confocal microscopy is considered the gold-standard of optical microscopy. It can be used to detect signals only coming from fluorescence photons and block noisy light, improving image resolution. It also has sectioning capabilities that making it useful for imaging dense tissue such as the brain (Naredi-Rainer *et al.*, 2017).

Zebrafish used in the experiments were euthanized with tricaine under IACUC protocol and then treated overnight in 4% paraformaldehyde (PFA). Two rinses of 1X PBS buffer were then used for PFA removal and brains were dissected under a dissection microscope with surgical tweezers. Brains were treated for a minimum of one night in CUBIC I to increase visibility and preserve fluorescence. They were then placed on microscope slides with antifade reagent and cover slips for imaging. Three groups of fish were examined and imaged: GFP+/RFP-, GFP+/RFP+ (treated), GFP+/RFP+ (untreated) to assess the efficiency of drug treatment on neuron ablation.

Results

The Search for an Effective Protocol

The original concentration of 10mM MTZ in an overnight treatment had successful neuron ablation efficacy in zebrafish during the beginning trials of using the virtual behavior experiment. Its ability to sufficiently ablate neurons spontaneously failed. This has been a reoccurring problem in the scientific community, where MTZ protocols have not been reliable between labs even when shown proficiency in one.

We began examining alternative conditions of the MTZ treatment in *mnx1*:Gal4 zebrafish crossed with UAS: nfsB-mcherry zebrafish. The *mnx1* line has expression in spinal cord cells that should be easily accessible for pharmacological treatment. We tested different concentrations of MTZ, added DMSO, and used aluminum foil to produce dark conditions. Using confocal fluorescence microscopy, none of these conditions showed successful cell ablation compared to a control group with no treatment.

We then turned to NFP, a newer drug known to be an efficient substrate for NTR-mediated cell ablation. NFP showed a higher degree of effectiveness for cell ablation in the *mnx1*:Gal4 zebrafish than MTZ did compared to the no treatment condition (Figure 9). The zebrafish were treated in a 2.5uM solution of NFP in EM from a 2.5mM stock solution of NFP in DMSO. They were treated for a 24-hour period in dark conditions using aluminum foil to block the light.

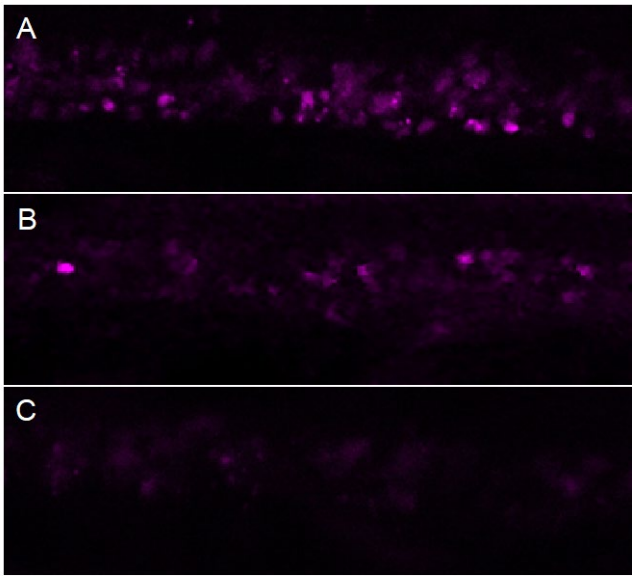

Figure 9: Pharmacological treatments in *mnx*1 zebrafish.

The density of cells expressing *nfsB*-mcherry protein is largely decreased after NFP treatment, suggesting efficient cell death. (A) No treatment; (B) 10mM MTZ, 24h; (C) 2.5 uM NFP, 24h

Analysis of Two Genetic Expression Patterns in Neurons

The *emx* cell-specific promoter produced over 10 lines of mutant zebrafish that each have their own unique expression pattern for neurons in the zebrafish brain. Of particular focus in this paper are two different expression patterns, referred to as EP #1 and EP #2. The important finding was the difference in measured social behavior after NTR prodrug treatments. The inactivity of EP #1 neurons did not disrupt normal social behavior, whereas after neuron ablation in EP #2 zebrafish had greatly decreased social behavior compared to the control group. Confocal imaging was used to assess the differences in expression.

EP #2 expression is widespread in the dTel and also incorporates clusters of neurons in the midbrain and hindbrain regions (Figure 10A). The EP #1 expression differs by having sparse expression in the dTel with unspecified regions of near the hindbrain and spinal cord (Figure 10B). The expression pattern differences can be assessed to make assumptions about which neurons are likely to be necessary in the development of typical social behavior. This would guide future research into elucidating distinct populations that are involved in the social behavior network.

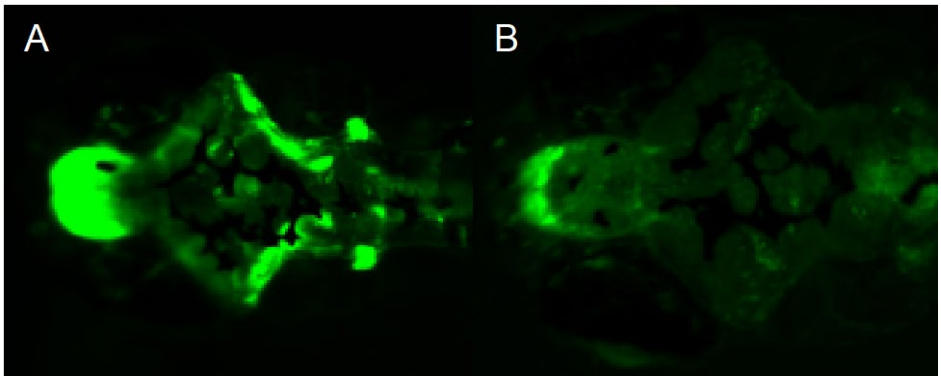

Figure 10: GFP expression differences in two *emx* lines.

(A) GFP expression in EP #2 is widely spread in the dTel and in areas of the midbrain and hindbrain. (B) GFP expression in EP #1 shows sparse expression in the dTel area and unspecified locations in the hindbrain and spinal cord.

Social Behavior

All lines of transgenic zebrafish that were tested using the virtual behavior assay were treated with either MTZ or NFP. The previously published *y321* transgenic line was tested as a positive control and novel *emx* lines with varying expression patterns were tested. The EP #2 of the *emx* line showed distinct behavioral phenotypes between

the RFP+ (experimental) and RFP- (control) groups after treatment in MTZ. The RFP+ group exhibited a decreased social index (SI) compared to the control group (Figure 11B), suggesting that the neuron populations expressed by this line are essential for the development of typical shoaling behavior. In comparison, EP #1 zebrafish showed no substantial difference in the behavior after neuron ablation (Figure 11A). The RFP+ SI was almost identical to that of the control group.

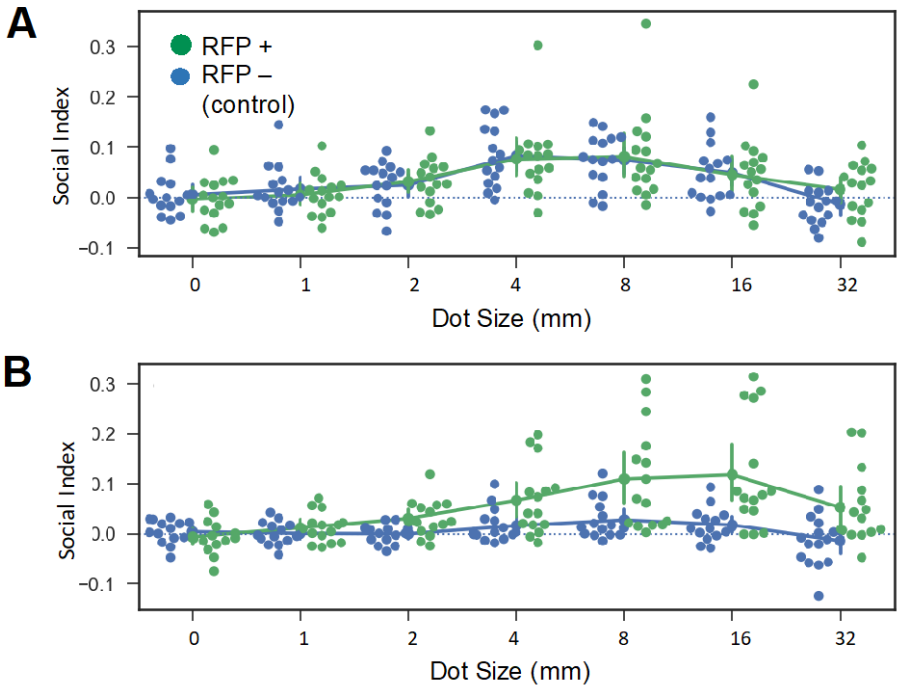

Figure 11: Social index versus dot size for two *emx* expression patterns.

Social behavior was measured after 10mM MTZ treatment in (A) EP #1 and (B) EP #2.

Zebrafish with EP #2 were also tested in the virtual behavior assay after 24h treatment with 2.5uM NFP. We found that the SI of the RFP+ group was noticeably

higher than the control group at most dot sizes (Figure 12). The errors bars show that at dot sizes 1, 2, 4, and 32 might not be significantly difference, but at dot sizes 8 and 16 there is significant differences in SI of both groups. In addition, the best fit line of the control group looks more similar to the baseline graph (Figure 6) compared to that of the control group after MTZ treatment (Figure 11B), suggesting it had a less toxic effect on the zebrafish than MTZ.

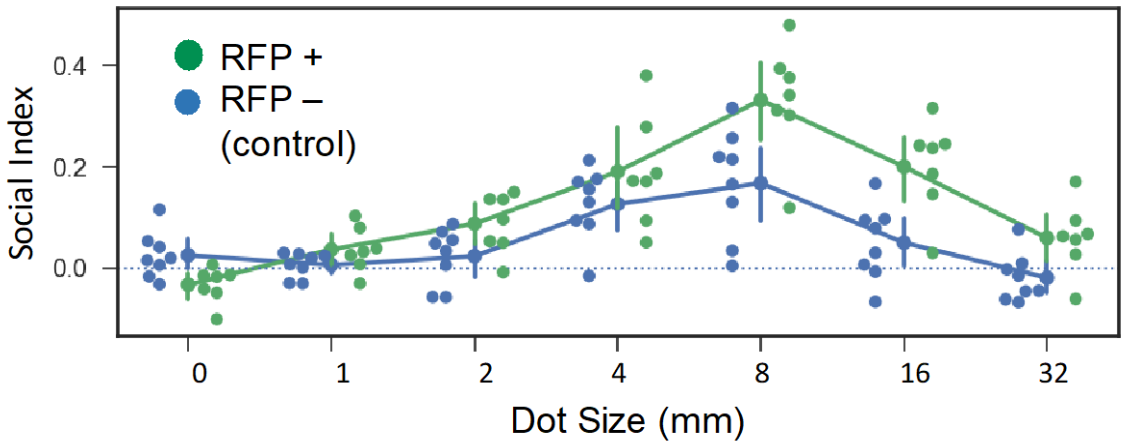

Figure 12: Social index versus dot size of EP #2 after NFP treatment.
The graph illustrates the best fit lines for social behavior outputs of RFP+ and RFP- groups after 24h treatment in 2.5uM of NFP and 24h recovery.

Controls

Virtual Dots

The use of unilateral interactions with virtual dots provide an environmental and behavioral control for individual zebrafish subjects. Having consistency among the environmental conditions in individual shallow dishes and among behavioral stimuli

(two-dimensional moving black dots), reduces possible confounding variables that may arise. Bilateral social interactions between two zebrafish are more complex because their behavior includes more social factors in addition to biological motion cues. The virtual dots only initiate shoaling behavior through the presence of simple biological motion cues that remain consistent for each individual subject.

The social indices displayed in the experimental graphs show similar trends seen in the baseline graph of social affiliation relative to the size of the projected dot illustrated in Figure 6. The baseline social indices at each dot size provides a control, particularly in examining if trends remain constant at the extreme ends of the graph (no dot stimulus; small and large dot stimuli).

Activity Levels

Another control for social behavior in the virtual behavior experiments is the activity level of each zebrafish, measured as their average speed. The average speeds of both RFP+ and RFP- groups were similar in both the EP #2 and EP #1 social experiments (Figure 13A,B). This reduces the chance that the deficits in social behavior observed in the EP #2 social experiments are not attributed to locomotion variables since both groups of treated zebrafish, those with ablated neurons and the control group had high levels of activity (Figure 13A).

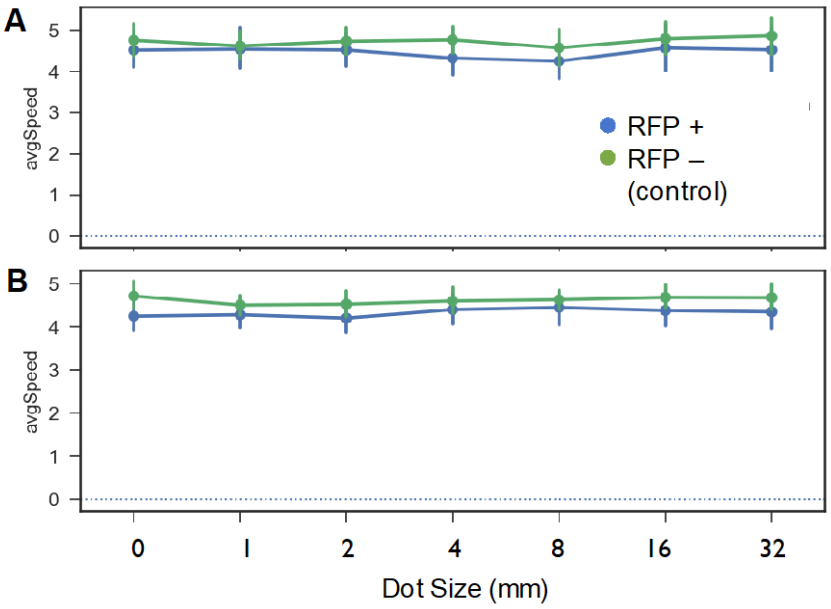

Figure 13: Average speed versus dot size during virtual behavior experiments.

Average speed (avgSpeed) for the RFP+ and RFP- groups of zebrafish at each projected dot size during virtual behavior experiments after 24h 2.5uM NFP treatment for EP #2 (A) and EP #1 (B).

Thigmotaxis Behavior

Another control for confounding variables of the virtual behavior experiments is tracking the thigmotaxis behavior of the zebrafish in each individual dish. Like shoaling, thigmotaxic behavior is another evolutionarily conserved behavior and develops by at least 5 dpf. It is a measure of anxiety-like behavior characterized by "wall-hugging" in novel environments (Shnorr *et al.*, 2011). In our behavioral assay, it can be used to ensure that any differences measured in social behavior between RFP+ and RFP- groups are influenced by differences in their thigmotaxis behavior. We found that the thigmotaxis behavior during virtual behavior experiments in EP #2 (Figure 14A) and EP #1 (Figure 14B) was very similar in both RFP+ and RFP- groups. These

graphs illustrate a control of zebrafish positions in the shallow dishes, and similarities between RFP+ and RFP- groups ensures thigmotaxis behavior is not influencing social index results.

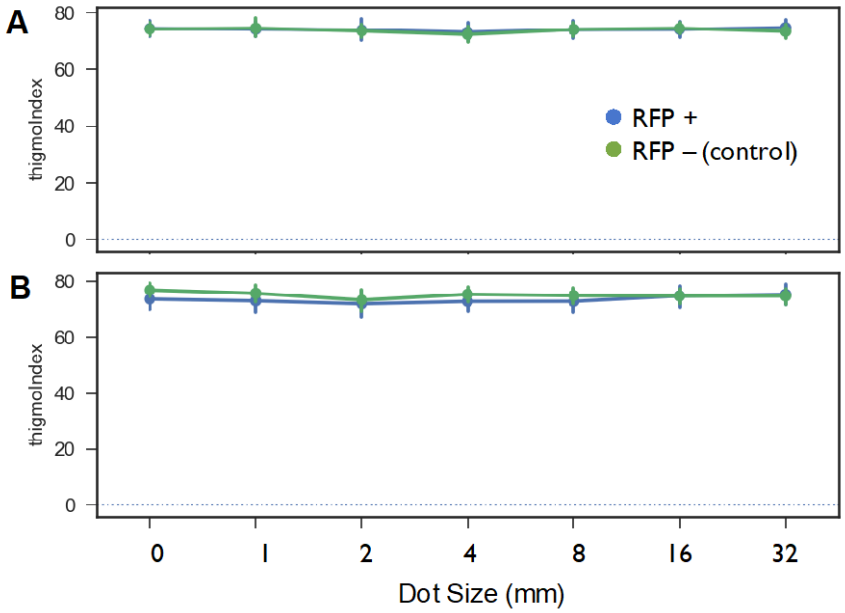

Figure 14: Zebrafish thigmotaxis during virtual behavior experiments.

Zebrafish thigmotaxis measurements of RFP+ and RFP- groups during virtual behavior experiments after 2.5uM NFP treatment; (A) EP #2 zebrafish and (B) EP #1 zebrafish.

Discussion

Improved Chemo-genetic Ablation Method

The previously used 10mM MTZ treatment no longer demonstrated efficiency in ablating neurons in the zebrafish brain. Switching to a 2.5uM NFP treatment improved NTR-mediated neuron ablation efficiency in our zebrafish larvae. Using bacterial NTR under cell-specific promotors is a newer genetic tool that has been useful for research in zebrafish regeneration and development processes. The prodrug that has been vastly used for this model is MTZ, however the common protocol use of 10mM has shown low reliability across research laboratories (Bergemann *et al.*, 2018). For Stednitz *et al.* (2018), the use of this protocol had a high efficiency, showing total ablation of neurons in zebrafish. Although, most research has found that this regimen is not efficient enough to ablate neurons, which are less easily accessible to pharmacological treatment than other cell types.

In our research, we wanted to establish a new protocol to make the NTR-mediated cell ablation model more efficient and reliable. Our protocol was successfully improved by switching the prodrug to NFP. These results confirm its previous success in recent research published in *Wound Repair and Regeneration* (Bergemann *et al.*, 2018) and suggests that, not only more efficient for our research, but a more reliable substrate than MTZ across research labs.

Newly Found Neuron Population in the Social Behavior Network

The overall goal of this project was to find a population of neurons in the developing zebrafish brain that is involved in the social behavior network. We were

able to discover and replicate how the inactivity of a population of neurons normally expressed in areas of the dTel, midbrain, and hindbrain, resulted in diminished social behavior activity. Our research showed that these results remained consistent when using either MTZ or NFP drugs, as long as they were efficient in actually inducing cell death.

Future Research

Future directions in this area will expand on using genetic and behavioral tools to narrow down the necessary neurons of the population discovered in the social behavior network of the developing zebrafish brain. Additionally, continued use of the nitroreductase-mediated cell ablation techniques with the virtual behavior assay will continued to be used to discover more neuronal populations in other transgenic zebrafish that have the ability to disrupt normal social behavior. Once populations can be elucidated to individual neurons involved in the social behavior network of the zebrafish brain, the genetic and behavioral roles of them in zebrafish and their association with the human brain will be further studied in developmental neuroscience.

Bibliography

Annaz, Dagmara, Campbell, Ruth, Coleman, Mike, Milne, Elizabeth, and Swettenham, John. "Young Children with Autism Spectrum Disorder Do Not Preferentially Attend to Biological Motion." *Journal of Autism and Developmental Disorders* 42.3 (2012): 401-08. Web.

Bergemann, D., Massoz, L., Bourdouxhe, J., Carril Pardo, C., Voz, M., Peers, B., & Manfroid, I. (2018). Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations. *Wound Repair and Regeneration, 26*(2), 238-244.

Courchesne, Campbell, & Solso. (2011). Brain growth across the life span in autism: Age-specific changes in anatomical pathology. *Brain Research, 1380*, 138-145.

De Polavieja, & Orger. (2018). Social Behavior: A Neural Circuit for Social Behavior in Zebrafish. *Current Biology, 28*(15), R828-R830.

Dobrucki, J. W. and Kubitscheck, U. (2017). Fluorescence Microscopy. In Fluorescence Microscopy, U. Kubitscheck (Ed.). doi:10.1002/9783527687732.ch3

Halpern, M., Rhee, J., Goll, M., Akitake, C., Parsons, M., & Leach, S. (2008). Gal4/UAS transgenic tools and their application to zebrafish. *Zebrafish, 5*(2), 97-110.

Larsch, & Baier. (2018). Biological Motion as an Innate Perceptual Mechanism Driving Social Affiliation. *Current Biology, 28*(22), 3523-3532.e4.

Lopes, Gonçalo, Niccolò Bonacchi, João Frazão, Joana P Neto, Bassam V Atallah, Sofia Soares, Luís Moreira, Sara Matias, Pavel M Itskov, Patrícia A Correia, Roberto E Medina, Lorenza Calcaterra, Elena Dreosti, Joseph J Paton, and Adam R Kampff. "Bonsai: An Event-based Framework for Processing and Controlling Data Streams." *Frontiers in Neuroinformatics* 9 (2015): 7. Web.

Lord, Elsabbagh, Baird, & Veenstra-Vanderweele. (2018). Autism spectrum disorder. *The Lancet, 392*(10146), 508-520.

Love, Alan C. "Functional Homology and Homology of Function: Biological Concepts and Philosophical Consequences." *Biology & Philosophy* 22.5 (2007): 691-708. Web.

Mathias, J., Zhang, Z., Saxena, M., & Mumm, J. (2014). Enhanced cell-specific ablation in zebrafish using a triple mutant of Escherichia coli nitroreductase. *Zebrafish,11*(2), 85-97.

Meshalkina, N. Kizlyk, V. Kysil, Collier, Echevarria, Abreu, . . . Kalueff. (2018). Zebrafish models of autism spectrum disorder. Experimental Neurology, 299(Pt A), 207-216.

Morita, Nitta, Kiyama, Mori, & Mishina. (1995). Differential expression of two zebrafish emx homeoprotein mRNAs in the developing brain. *Neuroscience Letters, 198*(2), 131-134.

Mueller, T. (2012). What is the thalamus in zebrafish? *Frontiers In Neuroscience, 6*(MAY), 64.

Naredi-Rainer, N. , Prescher, J. , Hartschuh, A. and Lamb, D. C. (2017). Confocal Microscopy. In Fluorescence Microscopy, U. Kubitscheck (Ed.). doi:10.1002/9783527687732.ch5

Nebel, Eloyan, Nettles, Sweeney, Ament, Ward, . . . Mostofsky. (2016). Intrinsic Visual-Motor Synchrony Correlates With Social Deficits in Autism. *Biological Psychiatry, 79*(8), 633-641.

Schnörr, Steenbergen, Richardson, & Champagne. (2011). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research, 228*(2), 367-374.

Stednitz, Mcdermott, Ncube, Tallafuss, Eisen, & Washbourne. (2018). Forebrain Control of Behaviorally Driven Social Orienting in Zebrafish. *Current Biology, 28*(15), 2445-2451.e3.

Sumbre, Germán, and Gonzalo G De Polavieja. "The World According to Zebrafish: How Neural Circuits Generate Behavior." *Frontiers in Neural Circuits* 8.JULY (2014): 91. Web.

Wang, Ying, Wang, Li, Xu, Qian, Liu, Dong, Chen, Lihong, Troje, Nikolaus F, . . . Jiang, Yi. (2018). Heritable aspects of biological motion perception and its covariation with autistic traits. *Proceedings of the National Academy of Sciences of the United States of America, 115*(8), 1937-1942.

Wullimann, Mario F., Rupp, Barbara, and Reichert, Heinrich. *Neuroanatomy of the Zebrafish Brain : A Topological Atlas*. Basel ; Boston: Birkhäuser Verlag, 1996. Print.