SYNAPSE DISTRIBUTION AND THE GUT MICROBIOTA IN *DANIO RERIO*

by

COLLETTE GOODE

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 2017

An Abstract of the Thesis of

Collette Goode for the degree of Bachelor of Science
in the Department of Biology to be taken June 2017.

Title: Synapse Distribution and the Gut Microbiota in *Danio rerio*

Approved: _____
Dr. Philip Washbourne

The human nervous system comprises billions of neurons. These cells transmit and receive signals from their neighbors via synapses. During synapse formation, hundreds of proteins localize to form connections between pre- and post-synaptic nerve terminals. Everyday behavioral and cognitive functions depend upon the proper formation and organization of synapses between neurons. Disruptions in synapse formation are known to correlate with neurodevelopmental diseases, including Autism Spectrum Disorder, Down's syndrome, and Tourette's syndrome. Studies in mice suggest that the microbiota can signal to the developing brain, indicating that changes in the intestinal microbiota may underlie some of the deficits seen in Autism Spectrum Disorder. The interplay between genetic factors and the gut microbiota underlying common neurodevelopmental disorders remain to be identified.

Danio rerio, the zebrafish, is an effective model organism in studying synapse formation. The first aim of this thesis applies immunohistochemistry and confocal microscopy techniques to characterize the distributions of various synaptic proteins in the developing zebrafish brain. The telencephalon was of particular interest, as this brain region is believed to correlate with complex behavior in zebrafish. This analysis found that Synaptotagmin 2b expression progressively increases in the telencephalon over development, possibly correlating with the onset of social behavior. The second aim of this thesis was to apply this descriptive analysis to analyze how the absence of bacteria in germ-free zebrafish affects synapse formation in the zebrafish brain. Using the synaptic markers from the first aim, we found synapse intensity and number to increase in the absence of bacteria, indicating that the gut microbiota controls synapse formation.

Acknowledgements

In completing this thesis, I am incredibly grateful for my education from both the Clark Honors College and the University's Department of Biology, as well as my invaluable research experience in the Washbourne Lab. I attended the University of Oregon with the intentions of obtaining a liberal arts education and lab research experience. Because of those named and the many not named in this section, I was successful in these aims. I first would like to thank Professor Philip Washbourne for granting me the opportunity more than two years ago to participate in the research of the Washbourne Lab. Beginning with zero research experience, this opportunity enabled me to develop the lab techniques, critical thinking, and collaboration skills necessary in the field of scientific research. Much appreciation to my mentor, Alex Tallafuss, for her guidance throughout this project—I have truly enjoyed our exploration of the zebrafish brain. I am also thankful for the opportunity to collaborate with Professor Judith Eisen on this project and observe her insight into research design and interpretation. This thesis also wouldn't have been possible without the support from present and past lab members. Furthermore, I thank the professors of the Robert D. Clark Honors College for promoting an interdisciplinary perspective to my undergraduate education. I would also like to thank the NIH funded Summer Program in Undergraduate Research at the University of Oregon for funding this project. Finally, I thank my family for their unwavering encouragement these past four years.

Table of Contents

List of Figures

List of Tables

Introduction

Neurodevelopmental Disorders on the Rise

Neurodevelopmental disorders (NDDs) are grouped by their early onset in development, usually by age three, but comprise varying symptoms. They are characterized by different impairments to social interactions, executive functions, speech and academic learning. Furthermore, NDDs can often co-occur, obscuring distinct diagnoses. Autism Spectrum Disorder (ASD) is one of the most common neurodevelopmental disorders, defined by a combination of abnormalities in speech, social interaction, abstract thinking and repetitive behaviors.[5,7] ASD wasn't recognized as a distinct disorder in psychiatric manuals of diagnosis until the 1980's.[7] In 2016, it was reported that approximately one in sixty-eight children have ASD. This estimate is about thirty percent higher than a previous estimate in 2012, reported one in eighty-eight children to have ASD.[5] The rising prevalence of autism and other neurological disorders motivates investigations into the possible underlying genetic and environmental factors.

Given the complexity of neurodevelopmental disorders, researchers are adopting a more holistic approach. In the field of science, the analytical "mechanistic" perspective is quite standard. Mechanism views the universe as a complex machine—one must identify each part of the machine independently to understand how the machine works, as described by philosopher Stephen Pepper. Mechanism is superior in its precision. However, its scope is limited, as biological systems are not as simple as machines[18]. While many factors have been identified in biological systems, researchers

must account for the potential unidentified factors remaining. In the case of neurodevelopmental disorders, there is no one clear cause. The significant increase in autism prevalence is partly due to increased awareness and changes in diagnostic criteria for autism. An extensive analysis of five million Californian births and 20,000 records from the state developmental services concluded that 15% of this increase is accredited to increased awareness and 25% due to diagnostic accretion.[30] Diagnostic accretion refers to children who formerly would have been diagnosed solely with mental retardation, but now are diagnosed also with autism. A remaining 46% of this increase is attributed to unknown causes.[30] Given the intricate nature of neurodevelopmental disorders, researchers now adopt a "contextual" approach, as described by Pepper. Contextualism accounts for the context of a situation, the interactions between numerous factors, and the inevitable disorder and unpredictability of the universe. The research field is shifting to recognize all the potential factors, investigating the interplay between social factors, genetic susceptibility and environmental factors in neurodevelopmental disorders.

The Gut Microbiota and Neural Development

In adopting the contextual view, there is currently much interest in the interactions between the nervous system and the microbial environment of the human intestine. The **gut microbiota** is the community of microorganisms co-inhabiting the human intestine, including bacteria, viruses, archaea, and eukaryotes.[14] While the human genome comprises 23,000 genes, the human microbiome contains 3.3 million genes.[20,25] It is even estimated that in a human, the number of bacteria surpass the

number of human cells.[15] The various microbial species harbor an array of symbiotic, commensal, and pathogenic interactions with a human host. Given the overwhelming presence of microorganisms in the human body, the research field recognizes the potential interplay between genetic and environmental factors. Because of public health implications, the National Institutes of Health launched the Human Microbiome Project in 2007 with the goal to characterize microbial species associated with human diseases. The prevalence of gastrointestinal issues, such as chronic constipation and diarrhea, in cases of ASD suggests an abnormal microbial composition underlying some neurological disorders.[10] This correlation instigated the hypothesis that the gut microbiota modulates neural development, particularly synapse formation, in the human host. Furthermore, a study in mice found that the absence of bacteria in mice increased motor activity and decreased anxiety.[8] It is of great interest to learn how the gut bacteria can elicit effects on behavior and neural development.

The Nervous System

The whole nervous system is subdivided into the **Central Nervous System** (CNS) and the **Peripheral Nervous System** (PNS). The CNS consists of the brain and spinal cord while the PNS includes all the peripheral nerves. The CNS integrates the information it receives from the peripheral nerves, and sends signals via the PNS to coordinate mechanisms throughout the body. This circuit depends on effective communication at the cellular level to enable proper autonomic, cognitive, and behavioral functions.

The human brain alone is estimated to contain 86 billion **neurons.**[1] Neurons are distinguished from other cell types by the projections that extend from their cell bodies: an **axon** and **dendrites**. Neurons receive signals from neighboring neurons through their dendrites and pass on information through their axons. The site of communication between neighboring neurons is known as a **synapse.** Among the one trillion CNS neurons, there are about one quadrillion synaptic connections.[1] Concentrated regions of dendrites, axons, synapses, but few cells, are referred to as neuropil.

There are both chemical and electrical synapses. **Electrical synapses** are built for speed. At these synapses, the pre- and post-synaptic cells are electrically coupled together, linked together by **gap junctions** so that ions can diffuse directly from one cell to the next to propagate an **action potential**, the depolarization of a cell's membrane. This instantaneous transmission mediates synchronization of many neurons for quick response such as reflexes or pacemakers. Studies also indicate that electrical synapses are crucial in the formation of chemical synapses.[19] Most synapses in the adult CNS use chemical transmission. While chemical transmission is slower than electrical transmission, its complexity enables a single neuron to amplify, modify, and compute different signals.[19] Chemical transmission begins with an action potential at the pre-synaptic cell. When the action potential reaches the pre-synaptic terminal, the depolarization causes channels to open, allowing an influx of calcium. At the **pre-synaptic terminal**, there are vesicles that contain one or more different neurotransmitters. In response to the presence of calcium, vesicles release neurotransmitters into the **synaptic cleft**, the space between the two adjacent cells. After release, neurotransmitters diffuse through the synaptic cleft and bind specific

protein receptors at the **post-synaptic terminal** to open ion channels.[19] A simplified diagram of a synapse between two neurons is depicted in Figure 1. In reality, hundreds of proteins localize at a chemical synapse to mediate synapse formation and functionality.[27]

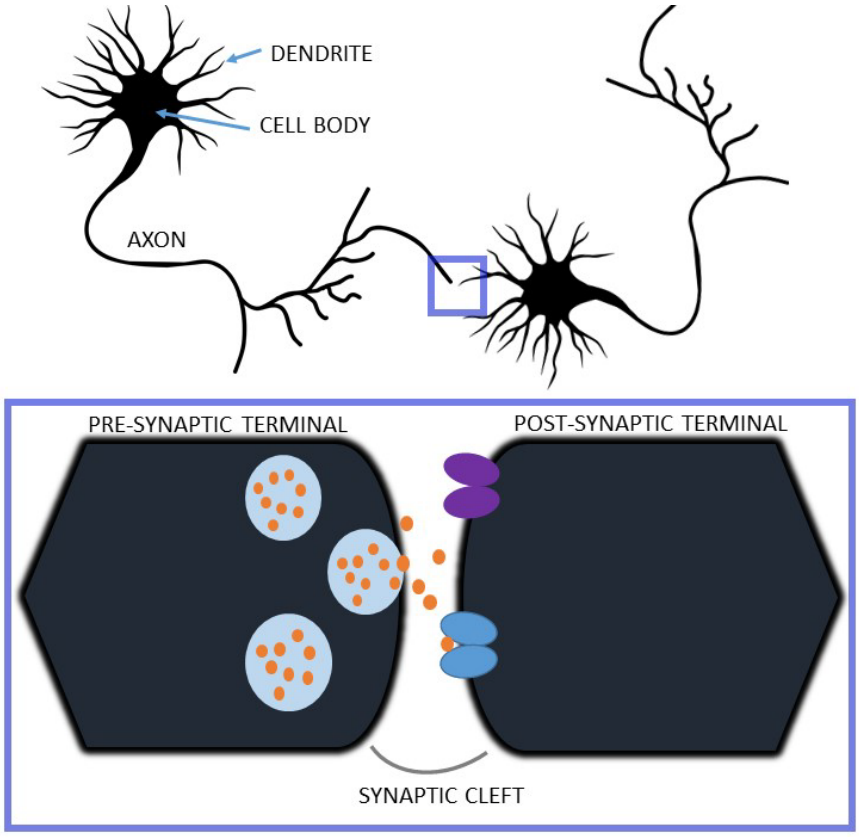

Figure 1: Structure of synapse between two neurons

Diagram of synapse between two neurons. Vesicles filled with neurotransmitter depicted at pre-synaptic terminal. Neurotransmitters released into the synaptic cleft. Receptors at post-synaptic cell to bind specified neurotransmitters.

Chemical synapses can be either inhibitory or excitatory. At **excitatory synapses**, the neurotransmitters released cause depolarization in the post-synaptic cell to increase the probability of an action potential. The most common excitatory

neurotransmitters are acetylcholine and glutamate. At the post-synaptic cell of an excitatory synapse, the **Glutamate receptor**—a glutamate-gated ion-channel—responds to the binding of glutamate at the post-synaptic cell.[24] The neurotransmitters released at **inhibitory synapses** cause hyperpolarization of the post-synaptic cell to inhibit the probability of an action potential. Serotonin and GABA are both examples of inhibitory neurotransmitters. At an inhibitory synapse, clusters of **Gephyrin** protein mediate fast hyperpolarization of the post-synaptic neuron.[22] A neuron can receive a combination of excitatory and inhibitory signals, and it is the balance of both these inputs that maintains homeostasis of the nervous system and other systems in the body.[21]

Danio rerio as a Model Organism

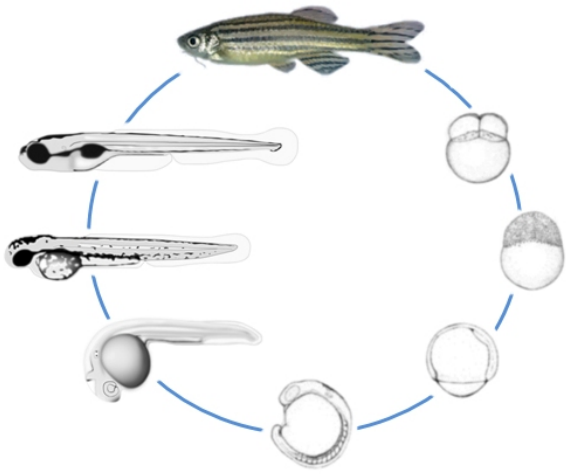

Figure 2: Life cycle of *Danio rerio*[29]

The zebrafish (*Danio rerio),* is an excellent **model organism** for studying synapse formation, brain development, and behavior. As vertebrates, zebrafish share

high homology with humans early in development. Gene programming is highly conserved in all vertebrates, as well as protein functions, cellular mechanisms, and some morphology. In the 1970s, optimization of genetic tools expanded the application of zebrafish as a model organism, implementing cloning, mutagenesis, and large-scale genetic screens.[22] Further, zebrafish are emerging as a prime model for neurological disorders and corresponding drug treatments. Now, many of the zebrafish mutant phenotypes identified in genetic screens are analogous to the symptoms of human disease states.[6] Zebrafish display quantifiable learning, sleep, drug addiction, and social behaviors.[2] The translucent tissues of zebrafish embryos also enable us to visualize brain development in a live animal. We can also achieve large sample sizes with this model organism, as one mating can generate hundreds of eggs. Zebrafish are also useful in studying how the microbiota might modulate synapse formation, brain development, and behavior because there is an effective protocol to raise “germ-free” zebrafish, or zebrafish without bacteria. Zebrafish, like humans, are colonized by bacteria present in the water after hatching. Studying germ-free fish enables us to analyze how the absence of bacteria affects synapse formation and behavior, and potentially to understand the relationship between gut microbiota and neural development.

Project Overview

Aim 1: Describing Neuropil Regions

The first aim of this thesis was to assess normal synaptic protein distribution over development in the zebrafish brain. The growing applications of zebrafish in neurobiology necessitates an understanding of zebrafish brain development.

Immunohistochemistry and confocal microscopy were used to label and visualize the distribution of synaptic proteins in the developing zebrafish brain and to identify individual synapses. The co-localization of a pre-and post-synaptic protein indicates a synapse. I analyzed the synaptic distribution in neuropil regions of the zebrafish brain at 6, 11, 14, and 18 days post-fertilization (dpf). I specifically imaged neuropil regions in the olfactory bulb (OB), telencephalon (Tel), diencephalon (Dien), optic tectum (TeO) and cerebellum (CCe), diagrammed in Figure 3.

I labeled and visualized the pre-synaptic proteins, **Synapsin 1/2**, **Synaptic vesicle glycoprotein (SV2)**, and **Synaptotagmin 2b (Syt2b)** in sagittal brain sections. The family of synapsin proteins are highly conserved among invertebrates and vertebrates, and known to modulate neurotransmitter at the pre-synaptic terminal. There is rising evidence that the synapsin protein family is also involved in synaptic vesicle docking, fusion, and recycling. Studies have also linked mutations in synapsin proteins and the onset of CNS pathologies, such as epilepsy and schizophrenia.[4] Synaptic vesicle glycoprotein (SV2) also resides in the membrane of synaptic vesicles and modulates vesicular release.[26] Finally, Synaptotagmin is another synaptic vesicle protein identified as a calcium sensor at the pre-synaptic terminal.[16, 28] I particularly studied the distribution of the Synaptotagmin 2b isoform (Syt2b). Syt2b's function as a calcium sensor is crucial for chemical transmission, so that neurotransmitter is effectively released from synaptic vesicles in response to the calcium signal.

Additionally, I labeled and visualized the two post-synaptic proteins, Gephyrin and Glutamate receptor 2/3 (GluR 2/3). Gephyrin is associated with inhibitory synapses while GluR 2/3 is associated with excitatory synapses. Gephyrin mediates the

hyperpolarization of the post-synaptic cell at an inhibitory synapse.[23] The co-localization of pre-and post-synaptic proteins indicates the presence of a synapse. The labeling of the specific post-synaptic proteins, Gephyrin and GluR 2/3, allows us to describe the distribution of excitatory and inhibitory synapses.

I also used the zn12 antibody, which recognizes the L2-HNK-1 carbohydrate expressed in primary neurons during early zebrafish development.[14] This immunolabeling enabled us to visualize morphology and axon pathways in the brain. I also used a transgenic zebrafish line, Tg(vglut2a:GFP), in which cells expressing glutamate transporter are labeled genetically with Green Fluorescent Protein (GFP).

Aim 2: Microbiota and Synapse Formation

The second aim of this thesis was to apply this qualitative description to analyzing how the microbiota modulate synapse formation in zebrafish.
We can identify protein functions in synapse formation by manipulating proteins in zebrafish lines by either increasing or disrupting protein function. We can also manipulate the environment, as in the case of germ-free zebrafish. To effectively analyze the effects of these manipulations, it is crucial to understand the normal morphology and distribution of neuropil regions in the zebrafish brain.

I used the synaptic proteins studied in my first aim to label and quantify synapse number and intensity in **germ-free** zebrafish that are raised in the absence of bacteria.

Materials and Methods

Overview

The two main methods used in this thesis are immunohistochemistry and confocal microscopy. **Immunohistochemistry** is a method of using antibodies to label proteins of interest in biological tissue. Antibodies are normally produced by the immune system. However, researchers can produce antibodies to bind to specific proteins of interest. I applied primary antibodies to zebrafish brain sections to label specific synaptic proteins. I then applied secondary antibodies, each with a distinct fluorescent tag, that bind to the primary antibodies. These labeled proteins in brain tissues could then be imaged with a confocal microscope.

To address my first aim of describing neuropil regions in the zebrafish brain, I used these two methods to visualize the distribution of synaptic proteins. To investigate the role of the microbiota in synapse formation, I used the qualitative description to determine which synaptic proteins to label and quantify in germ-free zebrafish, in which intestinal bacteria are absent during development.

Wild-type zebrafish

Zebrafish were raised according to the regulations of the Institutional Animal Care and Use Committee (IUCAC). For the qualitative study, wild-type ABC/TU zebrafish were crossed to obtain offspring. Zebrafish embryos were euthanized at 6 dpf using 2 mL Tricaine per petri dish with 25 embryos.

Germ-free zebrafish

To investigate the role of the microbiota in synapse formation, conventionalized and "germ-free" zebrafish were obtained. ABC/TU fish were crossed to obtain wild-type offspring. The embryos are immediately put into antibiotic embryo medium. Around five hours post-fertilization, embryos are put into iodine for two minutes and then bleached for twenty minutes. The germ-free group is then raised in sterile tank water and fed sterile food. The control conventionalized group was kept in similar flasks and inoculated with fish tank water after bleaching so that they were exposed to the bacteria normally present in the fish tank water during development.

Embryo Preparation and Histology

For standard cryo-sectioning, embryos were fixed with 4% **Paraformaldehyde (PFA)** in 1x **Phosphate-buffered saline (PBS)** overnight to stop biochemical processes in the cells and inhibit tissue degradation. The fixation time was limited to overnight versus 24 hours to ensure effective antibody labeling. The next morning, the embryos were rinsed three times with PBS buffer and submitted to the University of Oregon Histology Lab for cryo-sectioning. Zebrafish brains were cut into 16 μm thick sagittal sections and mounted on Starfrost microscope slides. Cryo-sectioned tissue was stored at -20˚C with drierite drying agent to preserve the tissue sections until immunohistochemical staining is performed.

To optimize the labeling of the anti-GluR 2/3 antibody, I used a "fresh-frozen" protocol instead for preparing the embryos for cryo-sectioning. This protocol entails cryo-sectioning to be performed on non-fixed, frozen embryos. Cryo-sectioned tissues on slides were kept at -20˚C for no more than 24 hours, and then underwent a post-

sectioning fixation. Frozen slides were immersed in 4% PFA-PBS solution for eight minutes, then rinsed in PBS.

Immunohistochemistry

The cryo-sectioned tissues were retrieved from storage at -20˚C and brought to room temperature. The slides were immersed in PBS solution for three minutes to rehydrate the tissue sections, and excess liquid then removed. Using a PAP hydrophobic pen, a liquid barrier was drawn on each slide to encircling the cryo-sectioned tissue. This barrier allows sections to be incubated with liquid solution, without the solution dripping off the edges of the slides. The brain sections were incubated for one hour at room temperature with a block buffer solution containing 5% Normal Goat Serum, 2% Bovine Serum Albumin (BSA), 0.3% Triton, and PBS. This blocking step prevents non-specific antibody binding. While the tissues incubated with blocking solution, the primary antibody solution was prepared. Prior to this experiment, numerous Immunohistochemistry (IHC) trials were conducted with varying antibody concentrations to identify optimal antibody concentrations. The antibody concentrations used are shown in Table 1. After blocking, 250 μL of the prepared primary antibody solution was pipetted on each slide. Tissues were incubated with primary antibodies overnight at 4°C. The next morning, the slides were washed in 0.1% Triton-PBS solution six times over a period of 1.5 hours. Alexa-Fluor secondary antibody solution was prepared, using a concentration of 1:750. 250μL of secondary antibody solution was pipetted onto each slide. Tissues were incubated with secondary antibodies 2-4 hours at room temperature. Tissues were washed in 0.1% Triton in PBS six times over a

period of 1.5 hours to wash away any unbound antibodies. If the cryo-sectioned tissues seemed susceptible to damage, the slides were washed in 0.1% Tween-PBS instead. Excess liquid was removed and cover slips were mounted on slides with DAPI hard set mounting medium to protect the tissues. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA and allows visualization of the sectioned tissue structure. Cover-slipped slides were stored at 4˚C until imaged with confocal microscope.

Antibody	Correlating protein	Concentration
Gephyrin (rabbit)	Gephyrin protein (post-synaptic)	1:500
GluR 2/3 (IgG2a mouse)	Glutamate receptor (post-synaptic)	1:250
SV2 (IgG mouse)	Synaptic vesicular glycoprotein 2 (pre-synaptic)	1:1000
Synapsin 1/2 (rabbit)	Synapsin protein 1,2 (pre-synaptic)	1:250
Znp1 (IgG2a mouse)	Synaptotagmin 2b (pre-synaptic)	1:500
Anti-GFP (chicken)	GFP expressing cells in transgenic zebrafish	1:1000
Zn-12 (IgG1 mouse)	HNK-1 carbohydrate expressed by primary cells of several functional classes.	1:1000

Table 1: Optimized primary antibody concentrations for Immunohistochemistry

Left column: antibody, middle column: correlating proteins it binds to, right column: optimal concentrations for immunohistochemical protocol. The animal in which each antibody was made is indicated in parenthesis.

Confocal Microscopy

Confocal microscopy is an optical imaging technique that increases resolution and contrast of images by using point illumination and a spatial pinhole to eliminate out-of-focus light in tissue sections.[17] Images were taken on an inverted Nikon TU-2000 microscope with an EZ-C1 confocal system (Nikon) with either a 20x, 60x water immersion, or 100x oil immersion objective, or a Zeiss LSM5 Pascal confocal microscope with ZEN software with 20x or 63x oil immersion objectives. Images were taken with a medium pinhole and laser intensity of 30.0%. Image channels were arranged such that the 488 nm, 546 nm, and 633 nm lasers corresponded with green, magenta, and cyan staining respectively. For each experiment, gain levels were optimized to visualize antibody labeling of proteins, but limit saturation and excessive background noise. When imaging an entire set of sections for quantification, the gain, laser intensity, and pinhole conditions were optimized using a saturation indicator and not altered for the remainder of the imaging process.

Quantification of synaptic proteins in GF and CV zebrafish

Images of the optic tectum, and lateral, ventral, and dorsal telencephalon were taken at 100x magnification. We focused on the telencephalon region of the zebrafish brain, a region that is known to control behavior in vertebrates. Cropped sections of the 100x images were saved. Images of each separate channel were saved in grayscale bitmap format. Using the Image Pro Plus® (Media Cybernetics) program, puncta labeled by Synapsin 1/2, Gephyrin, and GluR 2/3 were counted. The intensity and number of these labeled grayscale puncta were quantified and uploaded from Image Pro

Plus® to an excel spreadsheet. Student t-test was used in the quantitative analysis of germ-free and conventional zebrafish.

Results

Aim 1: Describing Neuropil Regions in the Zebrafish Brain

The purpose of this study was to characterize neuropil regions to optimize analysis of germ-free and mutant zebrafish. I used immunohistochemistry and confocal microscopy to visualize the distribution of pre-and post- synaptic proteins in the developing zebrafish brain. The zebrafish brain shares similar structures to the human brain, however the forebrain in zebrafish is less developed. The zebrafish brain is much easier to study than the more complex human brain, and its homology with the human brain make it an applicable model for understanding neurodevelopmental disorders.[31] The optic tectum mostly mediates visual processing and plays a role in visual grasping and prey capture in zebrafish, while the olfactory bulb allows zebrafish to sense odors. Further, the cerebellum is important for sensory integration and motor control in zebrafish.[31] The telencephalon, which is particularly studied in this thesis, is speculated to correlate with social behavior in zebrafish, characterized by swimming near other fish. The brain structures of the olfactory bulb, telencephalon, optic tectum, and cerebellum are easily visualized in lateral or sagittal brain sections (Figure 3).

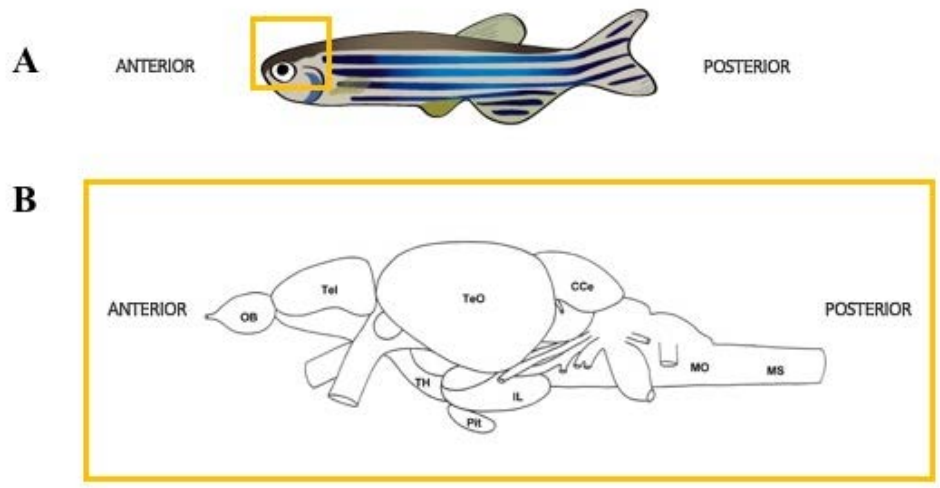

Figure 3: Sagittal diagram of the zebrafish brain

A) Lateral (sagittal) view of an adult zebrafish. **B)** Sagittal diagram of the zebrafish brain. Olfactory bulb (OB), telencephalon (Tel), optic tectum (TeO), and cerebellum (CCe) depicted. The hypothalamus (TH), pituitary (Pit) and inferior lobe (IL) all make up the diencephalon brain region.[11,31]

Pre- and Post-Synaptic Proteins in the Developing Zebrafish Brain

This analysis begins with the zebrafish brain at 6 dpf. We chose to study this time point in germ-free fish because larvae don't need to be fed until they are 7 dpf. avoiding any bacterial contamination from food. I first visualized pre-synaptic markers in neuropil regions using Synaptic vesicle glycoprotein (SV2), Synapsin 1/2, and Synaptotagmin 2b (Syt2b) in sagittal zebrafish brain sections at 6 dpf (Figure 4A). Immunohistochemistry was also applied to sagittal brain sections at 6 dpf to visualize the post-synaptic proteins, Gephyrin and Glutamate receptor 2/3 (GluR 2/3). Gephyrin is associated with inhibitory synapses while the Glutamate receptors is associated with excitatory synapses. The resulting images display the distribution of effective post-synaptic markers at excitatory and inhibitory synapses in zebrafish brain sections at 6 dpf (Figure 4B).

The pre-synaptic markers SV2 and Synapsin 1/2 are broadly distributed in the telencephalon, optic tectum, diencephalon, and cerebellum neuropil regions. The post-synaptic marks GluR 2/3 and Gephyrin are share the same expression pattern of SV2 and Synapsin 1/2. All are prominently expressed in these neuropil regions (Figure 4B). However, Syt2b expression is barely detected in the forebrain (Figures 4A,4B). Syt2b is highly expressed in the optic tectum, hindbrain, and varied in the cerebellum.

The pre-synaptic proteins, SV2, Synapsin 1/2, and Syt2b were specifically imaged in the cerebellum neuropil region. While SV2 and Synapsin 1/2 are both distributed throughout the cerebellum, Syt2b expression is varied in this region (Figure 5). Both pre- and post-synaptic proteins were also imaged in the optic tectum. SV2 and Synapsin 1/2 are both broadly distributed throughout the optic tectum neuropil region. However, Syt2b is uniquely distributed in bands (Figure 6A). Gephyrin distribution appears to concentrate more in the dorsal optic tectum, while GluR 2/3 distributions is more highly detected in the ventral optic tectum (Figure 6B).

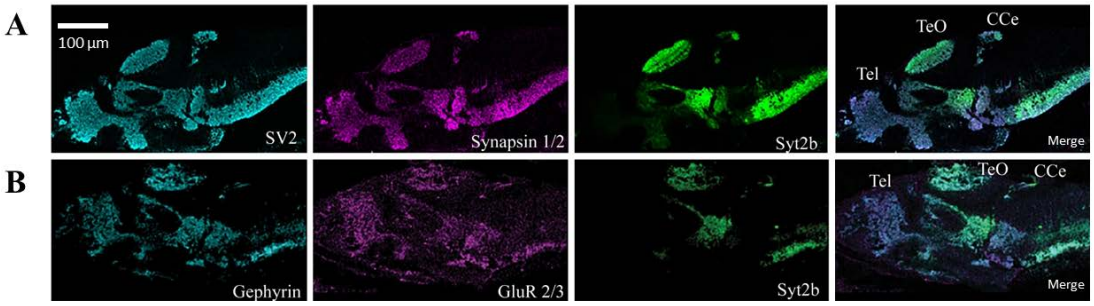

Figure 4: Distribution of pre- and post-synaptic proteins in zebrafish brain at 6 dpf

A) SV2 (cyan), Synapsin 1/2 (magenta), and Syt2b (green) labeled in sagittal brain section. Each separate channel shown, merge of all three channels on far right. **B)** Gephyrin (cyan), GluR 2/3 (magenta), and Syt2b (green) labeled in sagittal section. Each separate channel shown, merge of all three channels on far right. Imaged at 20x magnification. Scale bar = 100 µm.

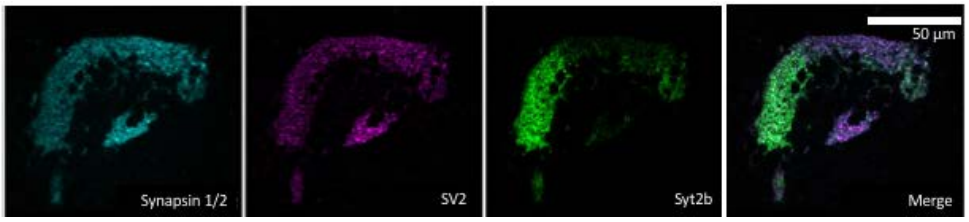

Figure 5: Pre-synaptic proteins in the cerebellum at 6 dpf

Distribution of Synapsin 1/2 (cyan), SV2 (magenta), and Syt2b (green), imaged in cerebellum in sagittal section of wild-type zebrafish 6 dpf. Imaged at 60x magnification. Scale bar = 50 µm.

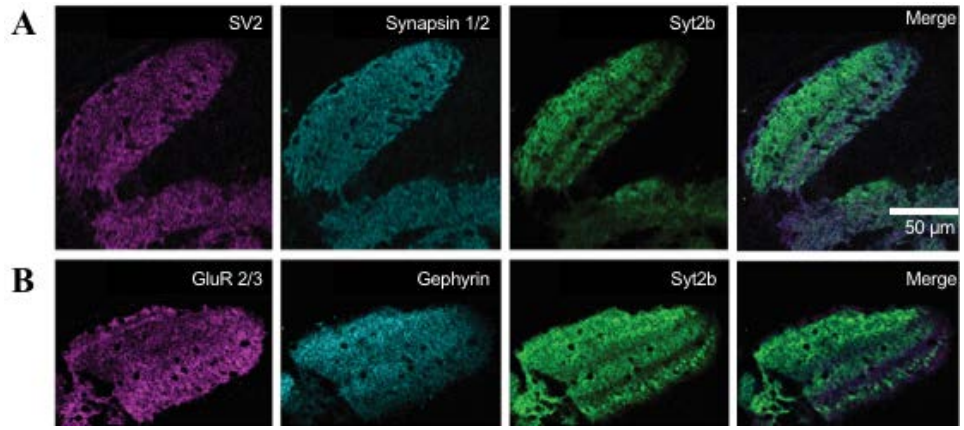

Figure 6: Pre- and post-synaptic proteins in the optic tectum at 6 dpf

A) SV2 (magenta), Synapsin 1/2 (cyan), and Syt2b (green) in optic tectum, sagittal section. Each separate channel shown, all three channels merged on far right. B) GluR 2/3 (magenta), Gephyrin (cyan), and Syt 2b (green), in the optic tectum, sagittal section. Each separate channel shown, all three channels merged on far right. Imaged at 60x magnification. Scale bar = 50 µm.

Synaptotagmin 2b Expression During Development

While the synaptic markers Synapsin 1/2, SV2, Gephyrin, and GluR 2/3 are all expressed in neuropil regions throughout the zebrafish brain, Syt2b is uniquely absent in the forebrain. Syt2b expression is also varied in the optic tectum and cerebellum. We took interest in Syt2b's distinct distribution, and sought to further investigate Syt2b distribution during development. I hypothesized that Syt2b expression in the forebrain increased through development. I looked at different time points during development to visualize the expansion of Syt2b labeling into more anterior forebrain regions.

Given zebrafish usually display social behavior around 12-14 dpf, I chose the time points 11, 14, and 18 dpf to identify if Syt2b expression correlates with the onset of social behavior. I also included the time point of 18 dpf to see if Syt2b expression continues to progress after the onset of social behavior. Sagittal brain sections of wildtype zebrafish at 11, 14, and 18 dpf were immunolabeled for Synapsin1/2 and

Syt2b (Figure 7). Because Synapsin 1/2 is distributed throughout telencephalon at 6 dpf, it was useful to compare its distribution to Syt2b.

Syt2b is progressively expressed more anteriorly (Figures 7,8). Syt2b progression in the telencephalon is easily visualized in Figure 7. By 18 dpf, Syt2b expression in the telencephalon is much more evident compared to its expression in the zebrafish brain 6 dpf (Figure 8A, 8B). Interestingly, Syt2b expression remains sparse in the dorsal telencephalon, but more pronounced in the ventral region of the telencephalon (Figure 8B).

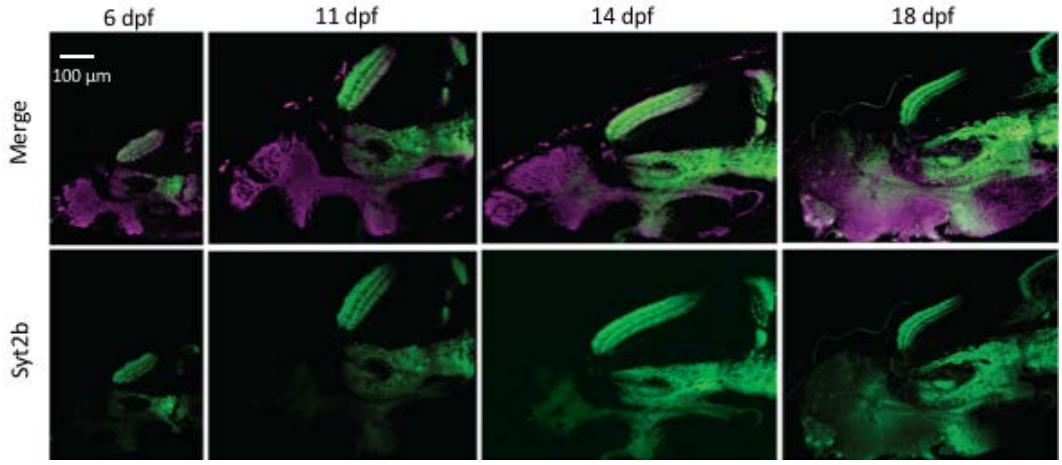

Figure 7: Synaptotagmin 2b expression in zebrafish brain over development

Syt2b (green) and Synapsin 1/2 (magenta) labeled in sagittal sections of zebrafish brains at 6, 11, 14, and 18 dpf. Upper panel: merge of both channels. Lower panel: Syt2b channel only. Scale bar = 100 µm.

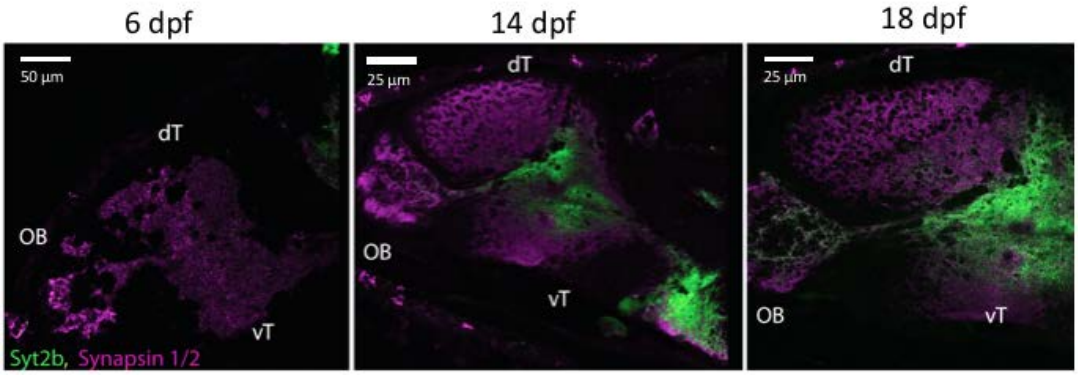

Figure 8: Progression of Syt2b in the telencephalon during development

Sagittal sections of telencephalon in zebrafish at 6, 14, and 18 dpf. Synapsin 1/2 (magenta) and Syt2b (green) labeled. Images taken at 20x magnification. Scale bars = 50 µm, 25 µm, and 25 µm, respectively.

Synaptotagmin 2b's Association with Post-Synaptic Markers

To further investigate the nature of this protein, I compared Syt2b co-localization with Gephyrin and Glutamate receptors. Syt2b is minimally expressed at the most posterior region of the telencephalon at 6 dpf, and therefore individual Syt2b puncta are easy to visualize. I obtained cropped regions of the telencephalon in sagittal brain sections of zebrafish 6 dpf and quantified synapses labeled by Syt2b, Gephyrin, and GluR 2/3. I found 82% of Syt2b puncta co-localized with Glutamate receptor, while only 51% of Syt2b puncta co-localized with Gephyrin (Figure 9).

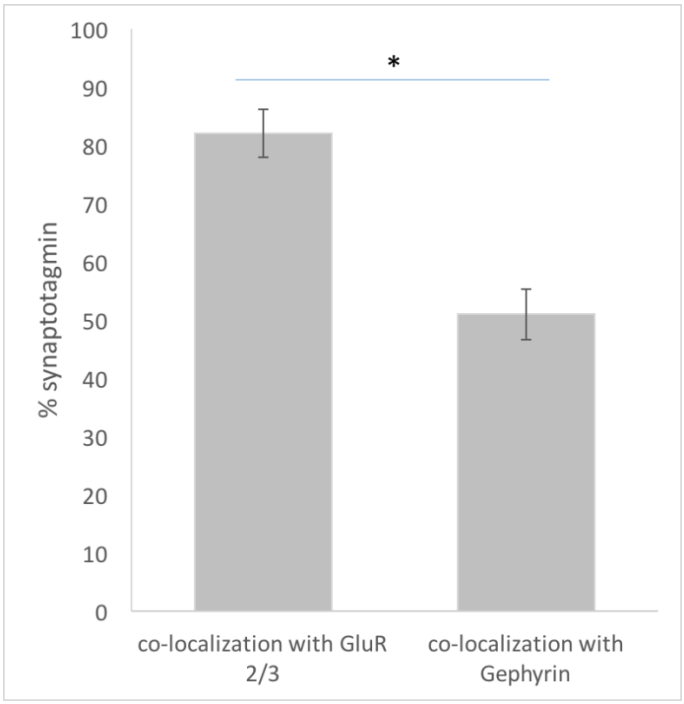

Figure 9: Syt2b co-localization with Gephyrin and GluR 2/3 in lateral telencephalon

82% of Syt2b puncta co-localizes with GluR 2/3 and 51% of Syt2b puncta co-localizes with Gephyrin. Student T-test. n=6. P-values < .0001.

Syt2b Located at Synapses in y321-GFP Positive Cells in the Ventral Telencephalon

To further characterize Syt2b, we studied its association with neurons in the ventral telencephalon using the transgenic zebrafish line *Tg(y321:gal4)* crossed to *Tg(UAS:GFP),* denoted y321-GFP. This transgenic line is used for social behavior experiments in the Washbourne Lab. Under a fluorescence microscope, a cluster of green cells can be visualized in the ventral telencephalon. This visualization enables lesions to be made in the ventral telencephalon area to study the role of the ventral telencephalon in zebrafish social behavior. I applied immunohistochemistry to label Syt2b and the GFP-expressing cells in y321-GFP zebrafish, and found a dense cluster of GFP neurons in the ventral telencephalon and some neurons in the optic tectum and hindbrain (Figure 9).

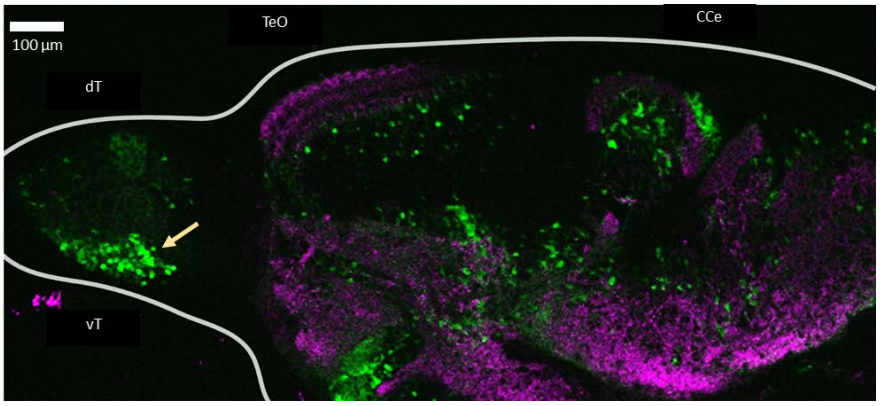

Figure 9: Sagittal brain section of y321-GFP Transgenic zebrafish

y321-GFP cells (green) and Syt2b (magenta) labeling in sagittal brain section imaged at 10x magnification. Cluster of y321-GFP cells in vT indicated by arrow. Dorsal telencephalon (dT), ventral telencephalon (vT), optic tectum (TeO), and cerebellum (CCe) labeled. Scale bar = 100 µm.

The telencephalon, particularly the ventral telencephalon, might be important for regulating social behavior in zebrafish. This transgenic line is useful for behavior studies and analysis of synaptic distribution in the brain. Given the correlation of the first visible Syt2b expression in the telencephalon and the onset of social behavior in zebrafish at 14 dpf, we used this line to further investigate the association of Syt2b with neurons in the ventral telencephalon.

Syt2b expression coincided with y321-GFP cells in both the ventral telencephalon and the optic tectum (Figure 10A and not shown). In fact, the Syt2b puncta perfectly co-localize with GFP labeled puncta. The GFP labeled puncta on the y321-GFP cells remain to be identified.

Because Syt2b progression in the telencephalon correlates with the onset of social behavior crica 12 dpf, I hypothesized that the number of Syt2b puncta on the y321-GFP cells increases during this period. To test this hypothesis, I quantified Syt2b

puncta on y321-GFP cells in the ventral telencephalon in zebrafish at 12 and 14 dpf. Number of Syt2b puncta at these cells increases during the time period of 12 and 14 dpf (Figure 11).

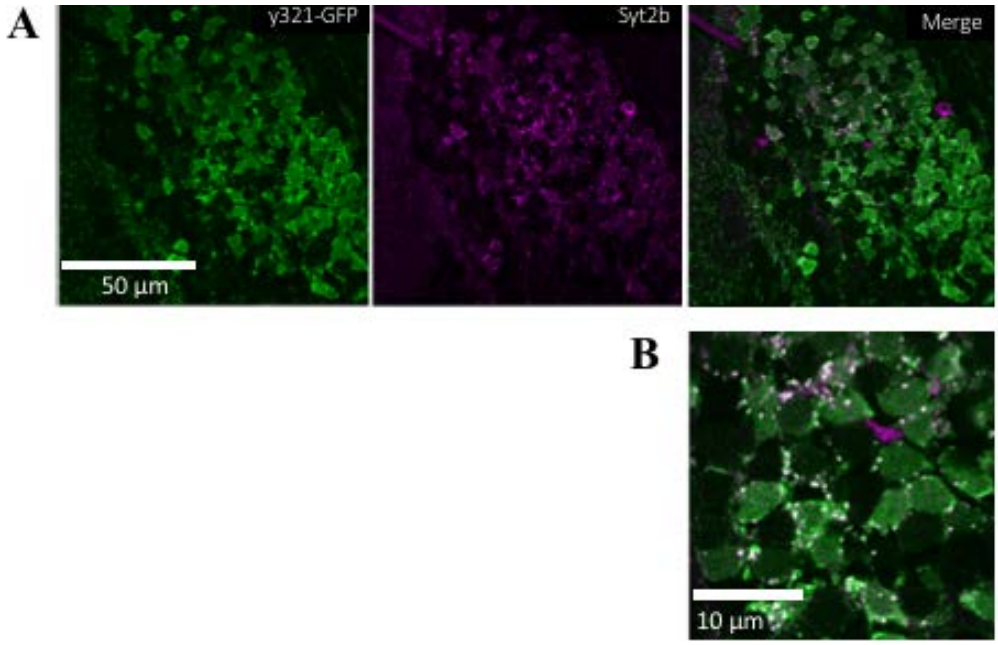

Figure 10: Syt2b labeling at synapses on y321-GFP cells in ventral telencephalon at 14 dpf.

A) y321-GFP cells (green) and Syt2b (magenta) labeled in the ventral telencephalon. Imaged at 63x magnification **B)** Syt2b puncta visualized at synapses with y321-GFP cells in the ventral telencephalon.

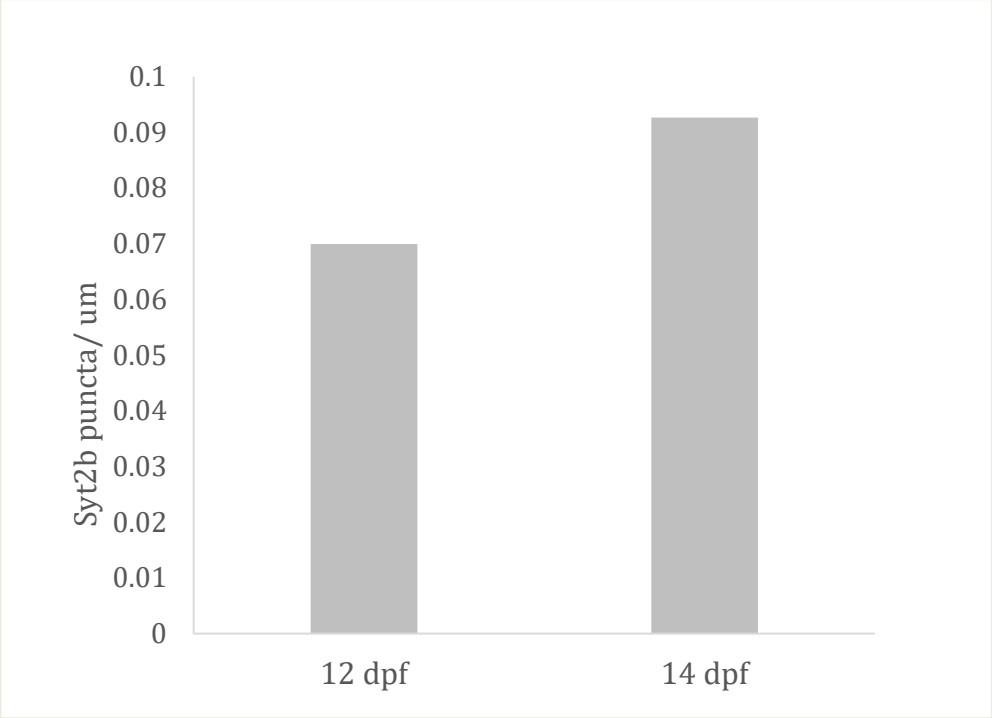

Figure 11: Syt2b puncta number at y321-GFP cells in ventral telencephalon during development.

Number of Syt2b puncta at y321-GFP cells in ventral telencephalon of zebrafish at 12 and 14 dpf. Student T-test. n=6. P-value = 0.056.

Aim 2: Microbiota and Synapse Formation

After establishing synaptic markers in the developing zebrafish brain, we asked if the absence of bacteria might affect the number of synaptic proteins.

Morphology of GF and CV Zebrafish

I used the pre- and post-synaptic proteins described in Aim 1 to visualize brain structure and synaptic protein distribution in germ-free and conventionalized zebrafish. I first looked at brain morphology and overall development of the germ-free (GF) and conventional (CV) zebrafish. We used a transgenic zebrafish line in which GFP is expressed under control of the vesicular glutamate transporter isoform 2a. Horizontal

brain sections of GF Tg(vglut2a:GFP) zebrafish were labeled with anti-GFP, Synapsin 1/2, and Gephyrin to visualize overall brain morphology (Figure 12A). Overall brain development, axon paths, labeled by zn12 antibody (green, Figure 13) and neuropil distribution appeared normal. There was also no obvious difference in overall development and size between germ-free and conventional zebrafish, other than the expected range of variation among individual zebrafish (Figure 12B).

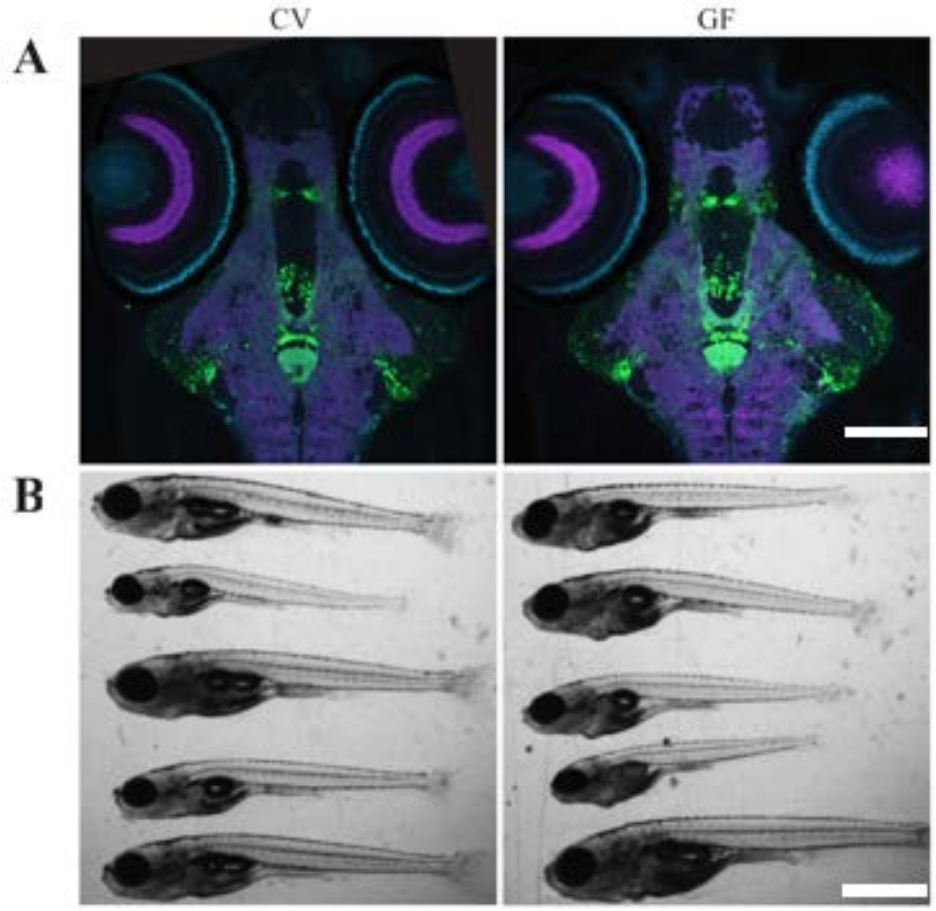

Figure 12: Morphology of GF and CV zebrafish at 6 and 19 dpf.

A) Vglut2a:GFP (green), Synapsin 1/2 (magenta), and Gephyrin (cyan) labeled in horizontal brain sections of GF and CV zebrafish at 6 dpf. Scale bar = 100 μm. B) Lateral images of GF and CV zebrafish at 19 dpf. Scale bar = 1 mm.

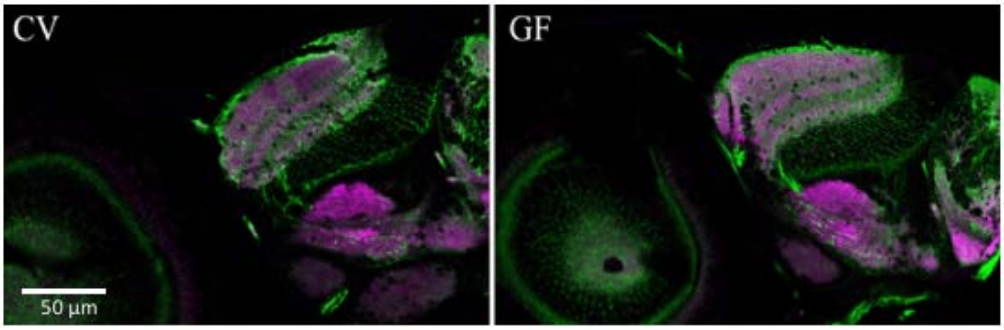

Figure 13: Neuropil and axon tracks in GF and CV zebrafish.

Primary axon tracks labeled with zn12 antibody (green) and neuropil regions labeled by Syt2b (magenta) in sagittal sections. Scale bar = 50 µm.

Synaptic Intensity and Density in GF and CV Zebrafish

I then applied immunohistochemistry and confocal microscopy to not only visualize germ-free and conventionalized zebrafish brain morphology, but to also quantify synaptic protein expression in neuropil regions of the brain. I analyzed the intensity of GluR 2/3 and Gephyrin labeling in neuropil regions of the optic tectum, lateral telencephalon, ventral telencephalon, and dorsal telencephalon in sagittal sections at 6 dpf. Intensity of Gephyrin expression was higher in the dorsal and lateral telencephalon and optic tectum in GF zebrafish. However, there was no significant difference in Gephyrin labeling intensity between GF and CV in the ventral telencephalon (Figure 14). GF zebrafish displayed higher intensity of Glutamate receptor expression in all measured neuropil regions compared to CV (Figure 15).

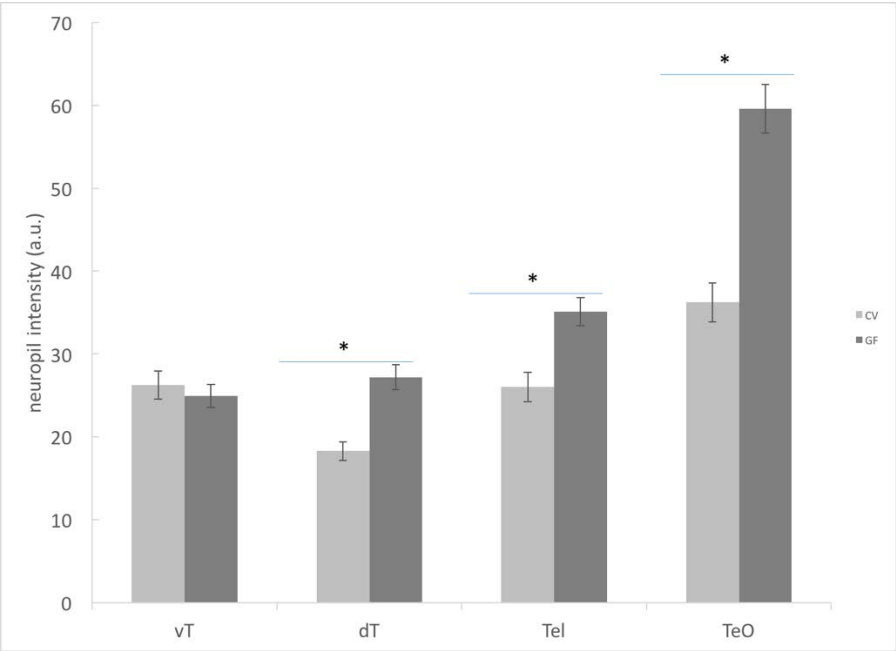

Figure 14: Gephyrin expression in neuropil regions in GF and CV zebrafish

Sagittal section of GF and CV zebrafish were imaged at 20x magnification. The intensity of Gephyrin labeling in neuropil regions was quantified in the ventral telencephalon (vT), dorsal telencephalon (dT), lateral telencephalon (Tel), and optic tectum (TeO). n=12. Student T-test. For vT: p-value = 0.55. For dT, Tel, and TeO: p-values < .0001.

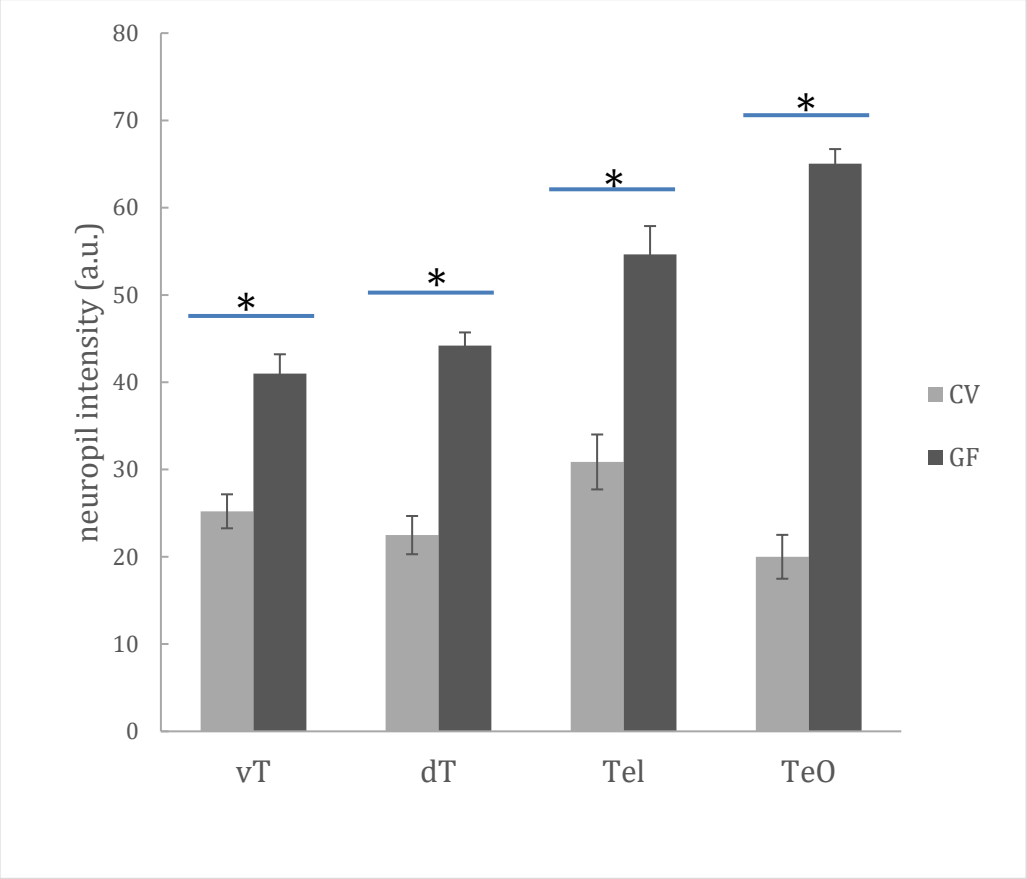

Figure 15: GluR 2/3 expression in neuropil regions in GF and CV zebrafish.

Sagittal section of GF and CV zebrafish were imaged at 20x magnification. The intensity of Glutamate receptor labeling was quantified in neuropil regions of the ventral telencephalon (vT), dorsal telencephalon (dT), lateral telencephalon (Tel), and optic tectum (TeO). n=12. Student T-test. P-values < .0001.

The increased intensity of synaptic protein labeling in neuropil regions of GF zebrafish can indicate either increased number of synapses, or increased localization of synaptic proteins at individual synapses. To test whether the absence of bacteria in GF zebrafish affected synapse number, I quantified density of synaptic proteins. I chose to analyze the lateral telencephalon region for synaptic density because there are some Syt2b puncta detected in this region at 6 dpf. I could use the labeling of the three synaptic

markers, GluR 2/3, Gephyrin, and Syt2b to quantify synaptic density in the telencephalon at 6 dpf (Figure 16). Puncta with intensities below the background noise were not counted, to normalize immunolabeling. GF zebrafish displayed higher number of Gephyrin, GluR 2/3, and Syt2b puncta in the telencephalon (Figure 17).

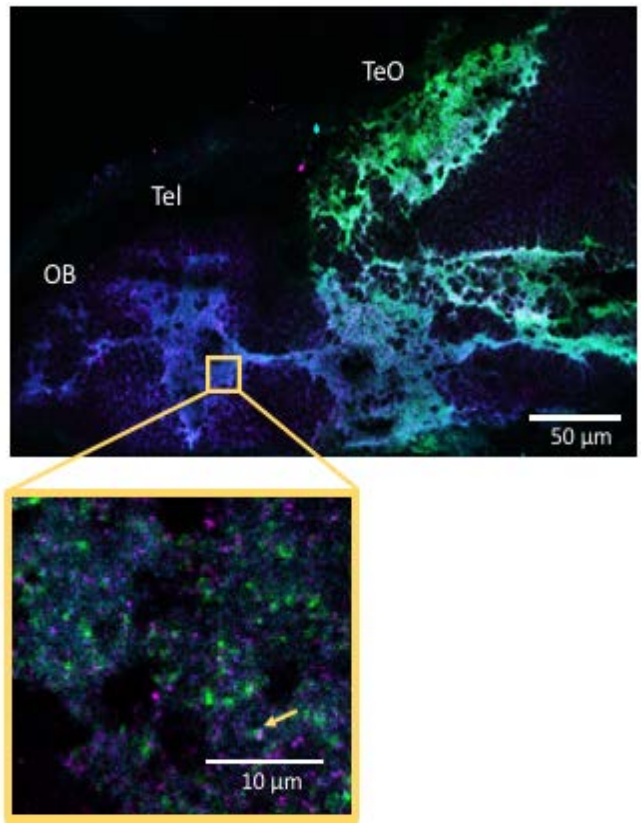

Figure 16: Puncta of synaptic markers in the telencephalon at 6 dpf.

A) Sagittal section of GF zebrafish brain at 20x magnification. Syt2b (green), Gephyrin (cyan), and GluR 2/3 (magenta) labeled. Scale bar = 50 µm. B) Puncta in telencephalon imaged at 100x magnification. Co-localization of markers indicate a synapse (shown by arrow). Scale bar = 10 µm.

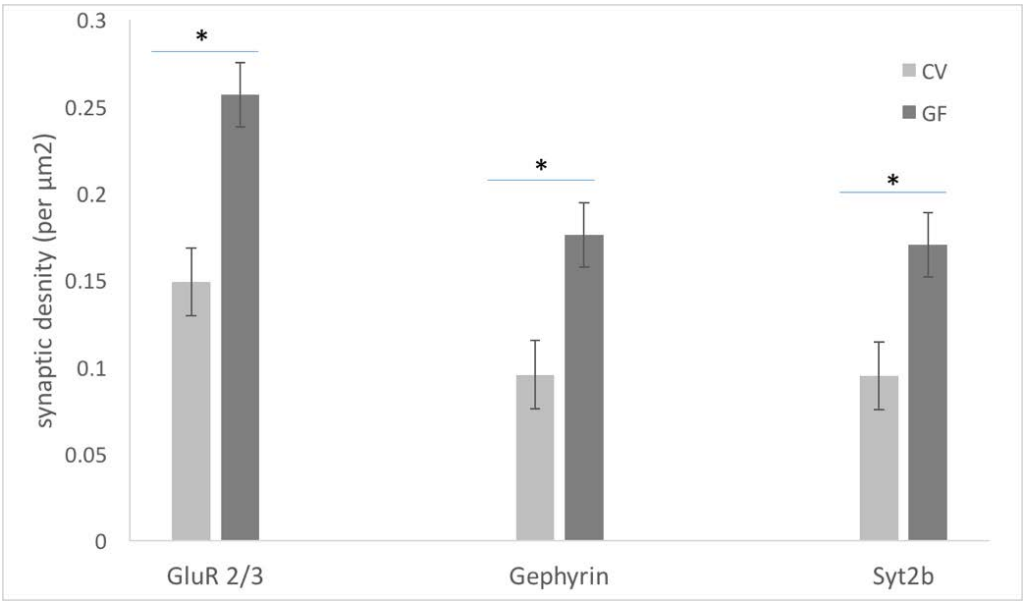

Figure 17: Number of synaptic proteins in GF and CV zebrafish

Synaptic density of each marker in lateral telencephalon of GF and CV zebrafish at 6 dpf. Student T-test. n=12. p-values < .01.

Discussion

Aim 1: Describing Neuropil Regions

This study identified the pre-synaptic markers, Synapsin 1/2 and SV2, to be prominently expressed in the neuropil regions of the telencephalon, optic tectum, cerebellum, and diencephalon. These markers are expressed by 6 dpf and can be used for analyzing synapse formation in GF or transgenic zebrafish at 6 dpf and older. The post-synaptic markers, Gephyrin and GluR 2/3, are also highly expressed in neuropil regions by 6 dpf. Both are distributed throughout the neuropil regions, as there appears to be no region in which one is expressed without the other. As Gephyrin is an inhibitory synaptic protein, and GluR 2/3 is an excitatory synaptic protein, these markers indicate equal distribution of inhibitory and excitatory synapses throughout neuropil regions in the zebrafish brain.

The progression of Syt2b in the telencephalon with development is of much interest. While expression at each time point wasn't quantified, it is visually apparent by immunohistochemistry staining and confocal microscopy that Syt2b expression progresses anteriorly into the telencephalon during the first 14 days of development. At 6 dpf, minimal Syt2b puncta are visible in the lateral telencephalon. By 14 dpf this protein is apparent in the telencephalon, and by 18 dpf, its expression progresses to the olfactory bulb (Figure 6,7). This synaptic protein's development is distinct from the other pre- and post-synaptic proteins of this study that are highly expressed in all neuropil regions in the brain by 6 dpf.

The correlation of Syt2b expression in the telencephalon and the onset of social behavior in zebrafish is certainly a phenomenon whose potential causal relationship should be further investigated. The telencephalon, particularly the ventral telencephalon, is possibly closely associated with complex social behavior in fish. Lesions in the ventral telencephalon in goldfish disrupted mating behavior and olfaction.[12] Furthermore, lesions in the ventral telencephalon in zebrafish also affect social behavior (Stednitz, unpublished).

By using the y321-GFP transgenic zebrafish, I was able to further investigate Syt2b's association with neurons in the ventral telencephalon region. I found the number of Syt2b puncta at y321-GFP cells to increase during the time period 12 and 14 dpf. I used the statistical analysis, student-test, obtaining a p-value of 0.056. While p-values less than 0.05 indicate data as significant, this analysis involved a small sample size of six zebrafish at each time point. These two data sets are also from time points only two days apart. I would predict a lower p-value if this analysis was conducted on data sets from more distant time points, such as zebrafish at 10 and 14 dpf. These synapses marked by Syt2b mark either the formation of new synapses or the localization of Syt2b at pre-existing synapses through development in this region. It would be interesting to label another pre-synaptic protein, in addition to Syt2b, in the y321-GFP line to investigate whether Syt2b marks new synapses or the increased complexity of existing synapses. Given the ventral telencephalon's potential role in behavior and the expression pattern of Syt2b, we plan to further study Syt2b's association with the y321-GFP ventral telencephalon cells.

Aim 2: Microbiota and Synapse Formation

We applied the identified markers from Aim 1 to investigate how the absence of bacteria in germ-free zebrafish affects synapse formation. As Synapsin 1/2, GluR 2/3, and Gephyrin are expressed in all neuropil regions by 6 dpf, we used these markers to analyze synapse formation in GF zebrafish 6 dpf. Since Syt2b is at least minimally expressed in the lateral telencephalon at this time point, we could quantify puncta of this marker as well. GF zebrafish showed increased synaptic density of the synaptic markers, Gephyrin, GluR 2/3, and Syt2b in the lateral telencephalon (Figure 16). This analysis indicates increased synapse number in GF zebrafish. It possible that the gut microbiota control synapse formation in the brain during development.

In my analysis of synaptic protein expressionin neuropil regions, I found GF zebrafish displayed higher expression of Glutamate receptor in the ventral, dorsal, and lateral telencephalon, and the optic tectum. Gephyrin expression also increased in neuropil regions in GF zebrafish, except in the ventral telencephalon. It is interesting that there was an observed increase of Glutamate receptor in the ventral telencephalon, but no significant change in Gephyrin expression. This may indicate a disrupted ratio of inhibitory and excitatory synapses in the ventral telencephalon. Nervous system function is maintained by a balance of inhibitory and excitatory signals, and imbalance to this system can be pathogenic. A study of epilepsy found that decreased inhibition caused hyperactive brain oscillation.[3] Furthermore, a study of Parkinson's disease found that an imbalance of excitation and inhibition in the brain impaired motor and cognitive functions.[13] It is proposed that an imbalance of excitation and inhibition also underlies neurodevelopmental disorders. My analysis of Gephyrin and GluR 2/3 expression in the

ventral telencephalon indicates that the gut microbiota might be important in establishing balance of inhibitory and excitatory synapses in the ventral telencephalon during development. However, it is crucial to account for variability in these complex biological system, as even wildtype conventional zebrafish display variability. The microbiota may affect synapse formation in varying regions in the brain among different organisms.

Conclusion

The descriptive analysis of Aim 1 in this thesis is useful for future studies in zebrafish neural development. Zebrafish is an exceptional model organism in this field, and the conclusions of Aim 1 can optimize future analyses. Future study of Syt2b's role in the ventral telencephalon and behavior could illuminate the factors driving the onset of social behavior in the developing zebrafish. Aim 2 exemplifies the application of this qualitative study in investigating the complex topic of microbiota and neural development. Using identified pre-and post-synaptic markers, we can study the potential genetic factors of NDD's in mutant zebrafish, as well as the microbial factors in germ-free zebrafish.

Appendix I: Glossary of Terms

Axon: is a long projection of a neuron that typically conducts electrical impulses away from the neuron's cell body.

Confocal Microscopy: an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. Also called confocal laser scanning microscopy.

DAPI: ',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used in fluorescent microscopy.

Dendrite: is a highly branched, generally tapering extension of a neuron that typically receives signals from other neurons and transmits the signals toward the cell body.

Excitatory synapse: A synapse in which the action potential in a pre-synaptic neuron increases the probability of an action potential occurring in the post-synaptic neuron.

Fixation: A chemical process by which tissues are preserved from decay. Fixation terminates any ongoing biochemical reactions, and increases the stability of the treated tissues.

Gap Junction: A small tubular protein structure at electrical synapses which allows for the movement of ions from one cell's interior to the next.

Gene: A molecular unit of heredity of a living organism.

Gephyrin: A post-synaptic protein, associated with inhibitory synapses, that mediates the hyperpolarization of the post-synaptic neuron.

Glutamate Receptor: A glutamate-binding ion channel at the post-synaptic terminal.

Immunohistochemistry: A method using antibodies to label and visualize specific proteins in biological tissue.

Inhibitory Synapse: A synapse in which a nerve impulse in the pre-synaptic cell results in a reduced probability for the post-synaptic cell to fire an action potential.

Knock-out mutant: A genetically engineered model organism in which researchers have inactivated, or "knocked out," an existing gene introducing a chemical that causes deletions in DNA.

Model Organism: A non-human species that shares common genes with humans. They are used to study biological phenomena, especially human diseases. Model organisms are employed when human experimentation is unfeasible or unethical.

Nervous System: The network of neurons and fibers that transmit signals and actions in the body.

Neuron: the functional cell of the nervous system.

Neurotransmitter: a signaling molecule in the nervous system.

Normal Goat Serum (NGS): used as a blocking reagent. It is effective in reducing nonspecific binding of proteins to reaction surfaces, thereby maximizing signal-to-noise ratio.

PBS: Phosphate Buffered Saline solution.

PFA: Paraformaldehyde, used as a fixative to inhibit degradation of proteins in biological living tissue.

Primary Antibody: A large, Y-shaped protein produced by the immune system used to identify, bind and neutralize pathogens such as bacteria and viruses. Researchers can use them to label proteins in biological tissue.

Secondary Antibody: A secondary antibody is an antibody that binds to primary antibodies or antibody fragments. They are typically labeled with probes that make them useful for detection, purification, or cell sorting applications.

Synapse: The junction and site of communication between two neighboring neurons in the nervous system.

Synaptic cleft: The space between two neighboring neurons at a synapse.

Synaptotagmin 2b: A synaptic vesicle protein at the pre-synaptic terminal, indicated to be calcium sensor.

Transgenic organism: A model organism that contains a gene or genes which have been artificially inserted via genetic engineering techniques.

Wild-type (WT): Wild-type refers to the phenotype of the typical form of a species as it occurs in nature. It is a normal “control” to compare a mutant organism, in which genes have been altered.

Bibliography

Azevedo, F. A.C., Carvalho, L. R.B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E.L., Leite, R. E.P., Filho, W. J., Lent, R. and Herculano-Houzel, S. "Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain." *The Journal of Comparative Neurology*. **513**.5. (2009): 532–541.

Best, J D, and Wendy K Alderton. "Zebrafish: An in Vivo Model for the Study of Neurological Diseases." *Neuropsychiatric Disease and Treatment* 4.3 (2008): 567–576. Print.

Brenner, R.P. "EEG in convulsive and nonconvulsive status epilepticus." *Journal of Clinical Neurophysiology* 21(2004): 319-321.

Cesca F., Baldelli P., Valtorta F., Benfenati F. "The synapsins: key actors of synapse function and plasticity." *Progress in Neurobiology.* 91.4 (2010): 313-348. Pubmed. Web. 20 Nov. 2016.

Demetriou, E. A, A Lampit, D S Quintana, S L Naismith, Y J C Song, J E Pye, I Hickie, A J Guastella. "Autism spectrum disorders: a meta-analysis of executive function." *Molecular Psychiatry* (2017) 00: 1-7. *Nature.com.* Web. 20 Nov. 2017.

Dooley, Kimberly, Leonard I Zon. "Zebrafish: a model system for the study of human disease." *Current Opinion in Genetics & Development.* 10.3 (2000): 252-256. ScienceDIrect. Web. 20 Nov. 2016

Geschwind, Daniel H. "Genetics of Autism Spectrum Disorders." *Trends in Cognitive Sciences* 15.9 (2011): 409–416. *PMC*. Web. 20 Nov 2017.

Heijtz, Rochellys, Shugui Wang, Farhana Anuar, Yu Qian, Britta Bjorkholm, Annika Samuelsson, Martin Hibberd, Hans Forssberg, Sven Pettersson. "Normal gut microbiota modulates brain development and behavior." *Proceedings of the National Academy of Sciences.* 108.7 (2011): 3047-3052.

King, Marissa, Peter Bearman. "Diagnostic change and the increased prevalence of autism." *International Journal of Epidemiology.* 38.5 (2009): 1224-1234. *Oxfordjournals.org.* Web. 22 April 2017.

Krajmalnik-Brown, Rosa et al. "Gut Bacteria in Children with Autism Spectrum Disorders: Challenges and Promise of Studying How a Complex Community Influences a Complex Disease." *Microbial Ecology in Health and Disease* 10.3402 (2015): 26900-26914. *PMC*. Web. 23 Nov. 2016.

Kreke, Nicola, Daniel R. Dietrick. “Physiological Endpoints for Potential SSRI Interactions in Fish.” *Critical Reviews in Toxicology.* 37 (2008): 215-47. *Pubmed.* Web. 22 April 2017.

Kyle, Ann, N.E. Stacey, R.E. Peter. “Ventral telencephalic lesions: effects on bisexual behavior, activity, and olfaction in the male goldfish.” *Behavioral and Neural Biology.* 36.3 (1982): 229-241.

Llinas, R.R., Ribary,U., Jeanmonod, D., Kronberg, E., Mitra, P.P. “Thalamacortical dysrhythmia: A neurological and neuropysychiatric syndrome characterized by magnetoencephalography.” *Proceedings of the National Academy of Sciences* 96 (1999): 15222-15227.

Metcalfe WK, PZ Myers, B Trevarrow, MB Bass, CB Kimmel. “Primary neurons that express the L2/HNK-1 carbohydrate during early development.” *Development.* 110.2 (1990): 491-504. Web. *PubMed.* 22 April 2017.

Methé, Barbara A. et. al. "A Framework for Human Microbiome Research." *Nature: International Weekly Journal for Science.* 486.11209 (2012): 215-21. Nature *Publishing Group.*13 June 2012. Web. 20 Nov. 2016.

O’Connor, Vincent, and Anthony G. “Synaptic vesicle fusion and synaptotagmin: 2B or not 2B? *Nature Neuroscience* 5.9 (2002): 823-824. *Pubmed.* Web. 1 May 2017.

Paddock, Stephen W., Thomas J. Fellers, and Michael W. Davidson. "Introductory Confocal Concepts." *Nikon's Microscopy*. Nikon Instruments, n.d. Web. 22 Nov. 2016.

Pepper, Stephen C. 1942. *World Hypotheses: A Study in Evidence*. Berkeley: University of California Press, 2015. Print.

Purves, Dale. "Chemical Synapses." *Neuroscience. 2nd Edition.* U.S. National Library of Medicine, 01 Jan. 1970. Web. 20 Nov. 2016.

Qin, J., R. Li, and J. Raes. "A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing." *Nature* 464.7285 (2010): 59-65. *Pubmed.org*. NCBI, 4 Mar. 2010. Web. 23 Nov. 2016.

Singer, Harvey J, Karen Minzer. “Neurobiology of Tourette’s syndrome: concepts of neuroanatomic localization and neurochemical abnormalities.” *Brain & Development* 25 (2003): S70-S84. Science Direct. Web 20 February 2017.

Streisinger, George, Charline Walker, Nancy Dower, Donna Knauber, Fred Singer. “Production of clones of homozygous diploid zebra fish (*Brachydanio rerio).*” *Nature 291 (1981): 293-296. Nature Publishing Group.* Web. 20 February 2017.

Tyagarajan, Shiva, Jean-Marc Fritschy. “Gephyrin: master reguluator of neuronal function?” *Nature Reviews Neuroscience.* 15 (2014): 141-156. *Nature.com.* Web. Nov. 2016.

Traynelis, Stephen F., Wollmuth, Lonnie, MBrain, Chris, Menniti, Frank, Vance, Katie, Ogden, Kevin, Hansen, Kasper. “Glutamate Receptor Ion Channels: Structure, Regulation, and Function.” *Pharmacological Reviews.* 62.3 (2010): 405-496.

Ursell, Luke K et al. “Defining the Human Microbiome.” *Nutrition reviews* 70.Supplement 1 (2012): S38–S44. *PMC*. Web. 28 Nov. 2016.

Vogl, Christian, Shota Tanifuji, Benedicte Danis, Veronique Daniels, Patrik Foerch, Christian Wolff, Banjamin J. Whalley, Sumiko Mochida, and Gary J. Stephens. “Synaptic vesicle glycoprotein 2A modulates vesicular release and calcium channel function at peripheral sympathetic synapses.” *European Jounral of Neuroscience.* 41.1 (2015): 398-409. *Academic Search Premier EBSCO host.* Web. 2 April 2017.

Washbourne, Philip. “Synapse Assembly and Neurodevelopmental Disorders.” *Neuropsychopharmacology* 40.1 (2015): 4–15. *PMC*. Web. 20 Nov. 2016.

Wen, Hua, Michael W. Linhoff, Mathew J. McGinley, Geng-Lin Li, Glen M. Corson, Gail Mandel, and Paul Brehm. “Distinct roles for two synaptotagmin isoforms in synchronous and asynchronous transmitter release at zebrafish neuromuscular junction.” *Proceedings of the National Academy of Sciences of the United States of America.*107.31 (2010): 13906-13911.

“Why zebrafish?” *FishforPharma*. Institute of Biology, Leiden University, 2015. Web. 20 Nov. 2016.

Wintraub, Karen. “Autism Counts.” *Nature* 479.7371 (2011): 22-24.

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