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Developing Sensory Behavioral Assays for Zebrafish Autism Model

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Developing Sensory Behavioral Assays for Zebrafish Autism Model

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By

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Under the mentorship of Vinoth Sittaramane

ABSTRACT

Individuals of all ages can suffer from a wide variety of symptoms and disabilities that could be diagnosed as Autism Spectrum Disorder (ASD). Due to new methods and technology, individuals are now being diagnosed in the first two years of their life, which is when the signs of ASD are initially exhibited. Individuals diagnosed with ASD share many similar disabilities and symptoms such as hyperactivity to social, visual, and auditory stimuli, as well as hyposensitivity to olfactory stimuli. Neural circuit-based alterations are widely considered as a cause for these behavioral aberrations. We have created behavioral assays using zebrafish larvae to study hyper-responsiveness towards social or visual stimuli, hyper-responsiveness with tactile/touch-based stimuli and remarkable hypo-responsiveness towards olfactory stimuli in autistic patients. This assay has provided baseline behavioral phenotype's for normal zebrafish larvae when exposed to the different stimuli. The baseline shows the distance moved, time spent, velocity, and movement patterns in the well for the normal zebrafish larvae. Moving forward we hypothesize that zebrafish autistic models will exhibit different phenotypes, which would allow us to screen for appropriate drug candidates for translation to human medicine. The variables that modify the zebrafish larvae's responses to the stimuli and their ability to learn the stimuli can be tested using these behavioral assays. Modifications that help regulate the behavior and responses of autistic zebrafish larvae can potentially be translated to autistic human patients. This could lead to improved treatment and medications for individuals with ASD.

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1. Background

Autism Spectrum Disorder (ASD) is a relatively new term for a wide range of disabilities and symptoms displayed in individuals of all ages. Early signs of ASD are seen in the first two years of an individual's life (8). Infants could show decreased eye contact or increased focus on certain objects. In the United States 1 in 68 children are diagnosed with ASD. It is still unknown what causes ASD, but there are speculations that it could be due to increased parental age, substance abuse amoung expecting mothers, vaccines, and many other possibilities (4). Many individuals diagnosed with ASD have similar characteristics such as hyperactivity to social, visual, auditory or tactile stimuli, and hyporesponsiveness to olfactory stimuli, etc. (1). Neural circuit-based alterations are widely considered to be a cause for these behavioral aberrations (11). It is imperative to understand the behavioral deficits and the neural defects underlying ASD in order to identify a therapeutic or diagnostic strategy for treating the disorder. In recent years, data has shown an increase in the number of individuals who are diagnosed with ASD; while this could be due to better detection, screening, and technology, it may also be due to an increase in the number of cases. Reproduction is evolutionary unfavorable in individuals with ASD, so it has not been known to be directly passed down one's lineage. More information is needed to explain why there is a higher number of autistic individuals around the world. Research must first be done on a comparable assay, such as zebrafish, which would allow back tracing of behaviors to neural circuits.

Professor Sittaramane's Lab has created genetic models of autistic zebrafish; however, the behavior and learning capabilities have not been properly evaluated due to a lack of consistent assays with regular zebrafish larvae. Over the past three years, I have

been designing behavioral assays and collecting data from wild-type zebrafish larvae when presented with different stimuli, such as visual or social stimuli, olfactory stimuli, and tactile stimuli. As I continue my research, I will be able to test these behavioral assays on autistic zebrafish larvae and compare the responses to wild-type larvae.

1.1 Brain Development and Genetics

Autism Spectrum Disorder has puzzled many researchers regarding the question; during what development stage do errors occur to result in ASD? ASD is classified as a "pervasive developmental disorder", meaning that it can disrupt different cognitive, behavioral, and developmental functions (13). It is found that individuals with ASD have a bilateral temporal hypoperfusion. This decreased blood flow in the temporal regions could be causing language impairment; this is different from a normal functioning temporal region which can effectively process words and images (14). It has also been shown that this could lead to impairments in social orienting, joint attention empathy, as well as face recognition; many of these impairments can be exhibited in children as young as one year of age (4). Genetics can also make a child more likely to be diagnosed with Autism. Monogenetic disorders, such as Rett's syndrome and Fragile X syndrome, are closely correlated with autistic individuals. Rett's syndrome which encodes the methyl-cytosine binding protein will eventually cause repetitive hand movements and a regression of neurological and social skills. Fragile X syndrome displays developmental delay and somatic dysmorphology (i.e. large forehead), which, once again, can be associated with Autism (14). In fact, it has been found that in individuals with Autism, regions of the brain that are vital to complex cognitive functioning, including attention,

social behavior, and language, enlarge during development, leading to altered function (2). However, it is important to note that research has found that no single gene accounts for ASD but rather multiple genes, each posing a different risk factor, collectively express this disorder. The greater the number of genes, the higher risk of developing the syndrome (5).

1.2 Behavior and Cognitive Control

Individuals with ASD vary from one to another in that they do not all have the same symptoms. However, there are many symptoms that are seen among most individuals with ASD. Common symptoms exhibited by individuals with Autism include reduced eye contact, withdrawn or aggressive, trouble communicating, and difficulty processing daily situations. These symptoms can be correlated back to the zebrafish assay's response with the different stimulus elicited. When individuals with ASD are faced with a tactile stimulus, in many scenarios they will become overwhelmed and try to avoid the stimulus. This is seen either with reduced contact, acting out, or even completely avoiding the stimulus by going into a separate area. When exposed to a social stimulus, individuals with ASD are likely to show reduced interaction. The most dramatic response can be when individuals are faced with a negative stimulus; ASD individuals are likely to dramatically act out compared to their normal behavior due to being overwhelmed and frustrated. BACB guidelines define a function for every behavior exhibited by an individual with Autism. The behavior could be due to four possible functions: attention, escape, sensory stimulation, and desire for an item.

1.3 Behavioral Deficiencies in Infants and Toddlers

With recent improvement in technology, the Autism Society tries to diagnose every child by the time they are three (8). However, symptoms can be seen at three months of age. All children have a set of milestones that parents should observe their child displaying throughout the first three years of their life. For instance, if the child:

- avoids making eye contact
- displays frustration or anger when being held or cuddled
- displays very dramatic reactions to the way things smell, taste, look, feel or sound
- does not smile on their own by five months old or laugh by six months old
- does not show interest and joy in games
- has language delays or displays echolalia
- does not recognize or show interest in new objects

If a child displays a combination of these behaviors it is very likely that they could be on the spectrum. These symptoms could lead to difficulty socializing and relating to other children, as well as difficulty when exposed to new stimulus. It is very crucial for the child to be diagnosed at an early age to start a behavioral intervention plan (8). Behavioral intervention plans are crucial for the proper development of the individual.

1.4 Behavioral Deficiencies in School Age Children

Children with ASD between the ages of three and 18 can display a wide range of behavioral deficiencies. Children may have:

- difficulty relating to other individuals or a lack of interest in other individuals

- difficulty expressing wants and needs using typical language. Leading to more outburst and frustration
- difficulty following instructions
- difficulty learning new topics and skills

Behavioral therapy can be completed to try to help the individuals cope with the symptoms. Behavioral therapy will focus on the function of the behavior to elicit the appropriate treatment. Most behaviors displayed by school age children follow the four functions of behavior states above.

1.6 Objectives and Hypothesis

Human ASD patients exhibit hyper-responsiveness towards social or visual stimuli, hyper-responsiveness with tactile/touch-based stimuli and remarkable hypo-responsiveness towards olfactory stimuli. We have designed behavioral assays using zebrafish. We hypothesize that autistic zebrafish models will exhibit similar phenotypes and will allow us to screen for appropriate drug candidates for translation to human medicine.

1.7 Preliminary Results of Future Studies

Table 1. Anticipated results from the various stimulus. The different control and treatment subtitles are listed in the far-left column (grey shading). The potential expected results are listed in the first row (blue shading). The symbol X signifies if the particular result is expected.

Treatment	Increased Distance Moved	Decreased Distance Moved	Increased Duration in Inner Social Zone (Yellow)	Decreased Duration in Inner Social Zone (Yellow)	After learning Increased Distance Moved	After learning Decreased Distance Moved
Control Visual Stimulus		X	X		X	
Autistic Visual Stimulus	X			X	X? If they learn?	X? If they learn?
Control Tactile Stimulus		X		X	X	
Autistic Tactile Stimulus	X		X		X? If they learn?	X? If they learn?
Control Olfactory Stimulus		X		X	X	
Autistic Olfactory Stimulus	X		X		X? If they learn?	X? If they learn?

2. Materials and Methods

2.1 Zebrafish

Researchers across fields have begun to notice the value of the zebrafish (Danio rerio) as a model organism, including for research concerning the neurodevelopmental causes of Autism. Few of the many benefits of using zebrafish include their low-cost maintenance, small size, rapid development, and long-life spans (6, 10). Use of zebrafish is also advantageous in that they can be used in a variety of techniques ranging from loss/gain of function methods to embryological assays, such as that used in this experimental study. It

can also be used in genetic screenings to analyze neural pattern formation of genes; this with other screens have produced many mutant fish with unique morphological, physiological, and behavioral phenotypes which provide opportunities for further future research. Zebrafish also make for good model organisms due to the conservation of zebrafish and mammalian brain development, structure, and function. Not only is the organization of the main sections of the brain highly conserved between these organisms, but zebrafish also share homology with humans in regard to the six major senses (vision, olfaction, taste, tactile balance, and hearing) as well as the sensory pathways. For instance, it has been found that second order motion perception can be observed in zebrafish larvae, despite the fact that in humans, it requires processing of complex visual information in the visual cortex. This structural and functional homology consequently demonstrates the conservation of cognitive processing. Additionally, genes that function in the production of neurons and axons are also comparable to those controlling similar processes in humans; therefore, zebrafish mutants can also be used to study axon pathfinding/formation mechanisms that control movement and behavior. These findings support the use of zebrafish to study the developmental causes of autism such as in this experimental study. Structurally and functionally homologous regions in the brain linked to human Autism in zebrafish allows for the use of zebrafish mutants with alterations in these regions to better understand and study this neurodevelopmental syndrome. Likewise, social activities in zebrafish can also be used to study behavioral changes that could be associated with Autism in humans (11).

Mutant zebrafish larvae will be used to test the differences between behavior. When top3β was removed, antibody staining of axons in the zebrafish larvae showed a reduced amount of integrity within the trunk of embryos 5dpf (Figure 1). The early development of the zebrafish depends on the connections and extensions of axons for proper signal transmission during action potential. Due to the lack of solid connections, one can infer that the proper signals released from the action potential may not be sent; the signals may not be as amplified or may fire the wrong targets. The normal larvae in Figure 1A, is seen with distinct neuron branching down the trunk. The mutant larvae in Figure 1B and 1C have a very scattered neuron pattern, these results demonstrate that top3β is crucial for proper development.

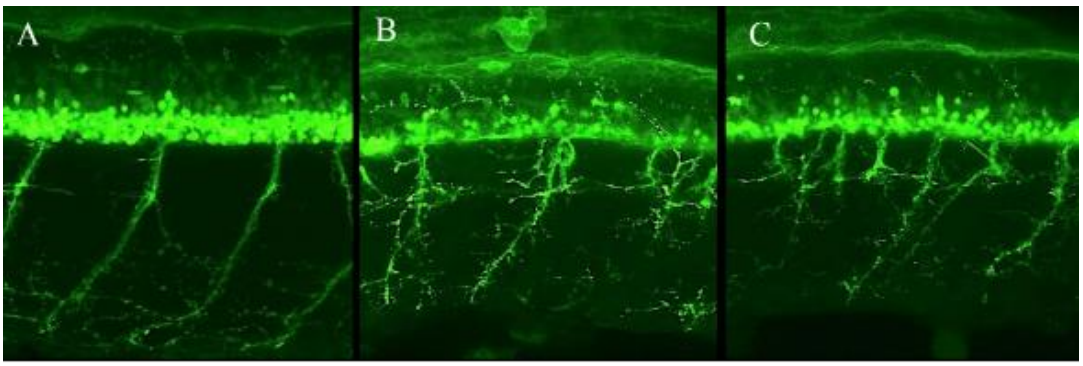

Figure 1. Antibody staining of axons in trunk of control (A) and morphant (B and C) embryos 5 dpf. Staining reveals a decrease in overall neurons, decreased integrity of neurons, abnormal branching patterns of caudal primary spinal motor neurons, and misconnection

2.2 Zebrafish Care

Zebrafish were held in a tank with the temperature between 26-28.5 °C. The room was light for 14 hours and dark for 10 hours. Embryos were checked on daily. When the zebrafish larvae were approximately one week old they were properly developed for the experiment.

2.3 Live Tracking

DanioVision and EthoVision software was used to track the movement of the zebrafish (Figure 2). A 6-well plate experiment was set up using the EthoVision software, and the plate was placed inside of the DanioVision. The experiment ran using a white light routine which included 30 minutes of light then 30 minutes of dark.

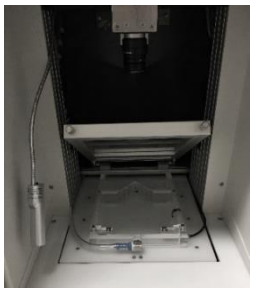

Figure 2. Image of DanioVision Observation Chamber

The zebrafish movement patterns were recorded using a white light routine. The EthoVision software was used to measure distance travelled in each zone, total time spent moving in each zone, and overall activity in the zones.

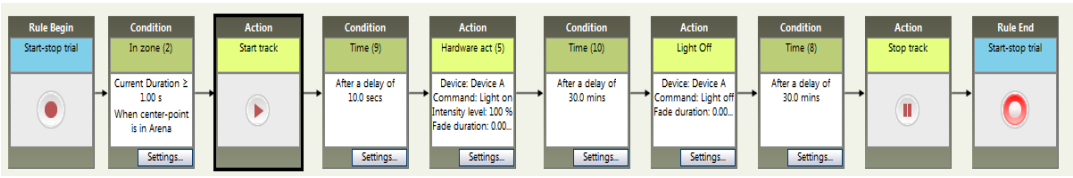

Figure 3. White light routine used for each experiment. The fish experienced 30 minutes of light and 30 minutes of dark.

2.4 Analysis of Live Tracking

After all the zebrafish tracking had been acquired, analysis of the data throughout the whole duration of the experiment was conducted. Each acquired well was analyzed separately to determine the total distance travelled, the average time moving in the well, and the velocity for each treatment and control group.

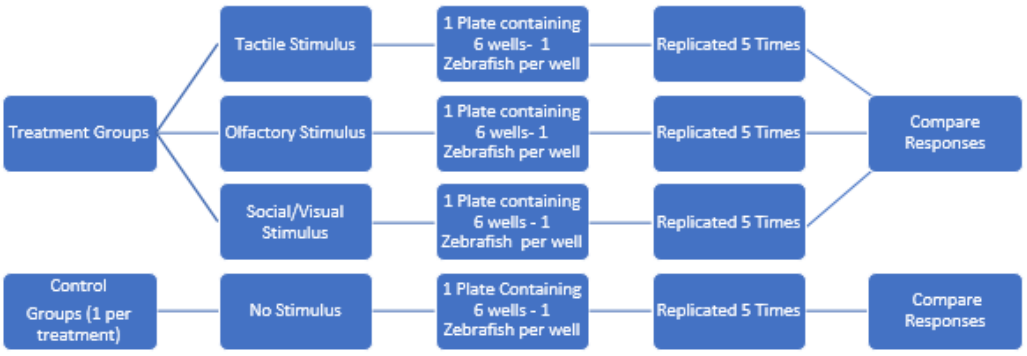

Figure 4 Experimental layout for each stimulus and control.

2.5 Experimental Design

A six-welled plate will contain one zebrafish larva per well. The well will contain an inner container that holds a visual/social, tactile, or olfactory stimulus. The visual/social stimulus will include 3 zebrafish larvae placed in the inner container. Visualizing other zebrafish larvae in the inner container will act as social stimuli for the zebrafish in the outer well to increase or decrease their movement and the amount of time spent closer to the inner zone. The tactile stimulus will have brine shrimp *(artemia)* placed in the inner zone and the brine shrimp will be able diffuse through the small holes in the inner container, creating a chaotic stimulus for the zebrafish larvae. This stimulus will trigger their gustation and tactile sensory receptors and cause the zebrafish in the outer well to move more or less.

The olfactory stimulus will have tissue from another dead zebrafish placed in the outer zone. This placement allows diffusion of dead fish odor and pheromones to reach the zebrafish placed in the outer well, thus causing the zebrafish to either move towards the dead zebrafish head or away from it (Figure 5).

Figure 5. The 6- well plate setup for the Olfactory stimulus. Pink outer circle represents the outer zone. The green middle region represents the middle zone. The grey inner zone represents the inner zone. The figure above displays a grey circle in the outer zone which shows the olfactory stimulus (dead zebrafish head).

The larvae will be exposed to the stimulus for one hour (30 minutes of light and 30 minutes of dark) to analyze light dependent behaviors. Using a DanioVision instrument and EthoVision software, the zebrafish movement patterns were recorded. The distance moved, duration spent, and movement pattern tracking in each of the social zones (Pink, Yellow or No Color zones in Figure 1, respectively) was recorded and the data was analyzed to find out the statistical significance of the data for control (wild-type) and Autistic zebrafish larvae. The experiments were performed and data from at least 30 larvae for each group was collected. There was a total of 6 zebrafish per trial (control and treated) and each trial was replicated 5 times. Thus, a total of 120 zebrafish was used for the experiment.

3. Results and Discussion

Individuals with autism spectrum disorder exhibit changes in neural circuitry of various regions of the brain that serve to function in perceptual processing. These affected portions include the primary cortical regions of the five sensory systems in the brain—vision, auditory, tactile, olfaction, and gustation. Neuroimaging evidence indicates that neural signatures of autism are present in early primary sensory regions of the brain in sensory processing of individuals with autism. Altered sensory perception in individuals with ASD has also been hypothesized to be linked to differences in neuronal circuitry in the brain. For instance, neuroimaging has been used to study whether the autistic sensory cortex may be marked by differences in GABA signaling pathways using mouse models; it was found that disruptions in GABA signaling as well as many other neurotransmitters and neuromodulators affected sensory perception in the autistic mice. These findings all support the idea that alterations in sensory symptoms in individuals with ASD can be used as phenotypic markers of autism. In fact, these differences can be visibly detected as early as 6 months into infancy; therefore, they could be useful in serving to predict diagnosis later in childhood, deficits in social function and cognition in adulthood, as well as alterations in neural circuitry in the primary cortical regions of the brain. These differences are also exhibited both in human and genetic animal models of ASD. Therefore, genetically modified animals can be useful in studying these changes in neuronal circuitry that result in sensory deficiencies in individuals with ASD (9). While mice have been known to be a useful model, zebrafish can also be used as models to study neuronal circuitry, defects and altered formations. Additionally, zebrafish make for good models for reasons stated above in section 2.1 Zebrafish (11). Although much

research has been done in identifying alterations in sensory perception in individuals with ASD, more is still required to have a better understanding on the neurobiology of autism and what specific differences make autism distinct from other neurodevelopmental diseases and disorders (1).

The results of this experiment display the behavioral phenotype of normal zebrafish larvae. These results are important in creating a baseline behavior to compare to autistic zebrafish larvae in future studies. The baseline behavior seen by the zebrafish in all three stimuli is that there is more movement in the outer well than in the middle well, which is the expected normal behavior among the larvae. The baseline behavior also shows that there is not a strong response to the different stimuli. The larvae might be able to adjust at a very quick rate to the different stimuli allowing them to not show a major response in behavior. In future studies, it is crucial to see the possible different behavioral phenotypes displayed by autistic zebrafish larvae. This baseline can be used as a standard of comparison moving forward to test different behavioral phenotypes.

3.1 Olfactory Stimulus

Throughout the White Light Routine, the embryo had increased distance moved, time spent, and velocity in the outer well compared to the middle well in both the control and treatment groups (Figure 6). However, there was no significant difference between the control group and treatment group exposed to the olfactory stimulus. For distance moved, analysis showed that the control group outer well and olfactory outer well were significantly larger than the respective middle wells with an adjusted P value less than 0.0001. Comparison of the distance moved between the control groups' outer/ middle

zone and the treatment groups' outer/ middle zone showed no significance (Figure 6A). Statistical analysis of the total duration spent in a zone showed that the control groups' outer well was significantly larger than the respective middle wells with an adjusted P value less than 0.0001. The treatment groups' outer well was significantly larger than the middle well with an adjusted P value of 0.002. Comparison of the duration between the control groups' outer/ middle zone and the treatment groups' outer/ middle zone showed no significance (Figure 6B). Analysis of the velocity between the groups showed no significance between treatment and control groups, as well as middle vs. outer zone (Figure 6C). The heatmaps from the experiment displays a large amount of time spent away from the olfactory stimulus located in the outer well of the arena. The heatmap does show that the embryo changed the normal expected behavior for a period of time to go into the middle well (Figure 6D).

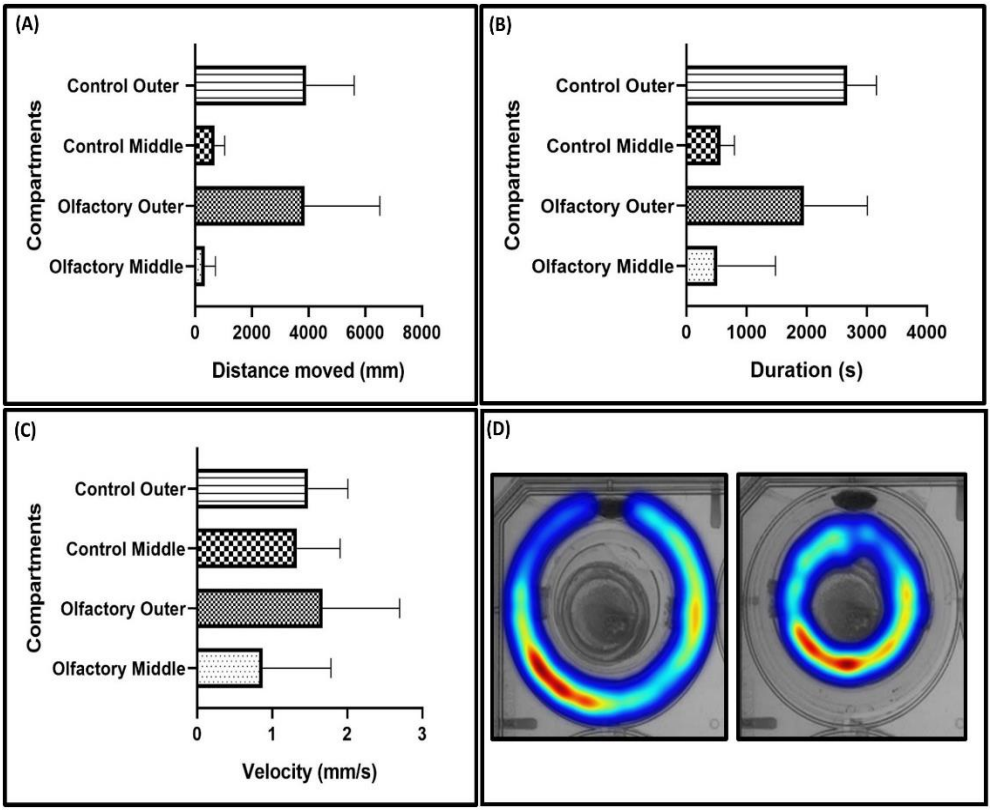

Figure 6. (A) The distance moved (millimeters) around the arena (outer and middle) for the control and treated group. Increased distance moved was seen in the outer well, but there was no significant difference seen between the two groups. (B) The cumulative time (seconds) spent moving around the arena (outer and middle) for the control and treated groups. There was increased time spent in the outer well, but there was no significant difference seen between the two groups. (C) The speed (millimeters per second) around the arena (outer and middle) for the control and treated groups. Increased velocity was seen in the outer well, but there was no significant difference seen between the two groups.

3.2 Tactile Stimulus

The addition of brine shrimp in the inner well for the tactile stimulus did not show a significant change in distance moved, time spent, and velocity between the treatment and control groups. The embryo was much more active in the outer well compared to the inner well in both the treatment and control groups (Figure 7). For distance moved, analysis showed that the control groups' outer well and treatment groups' outer well were

significantly larger than the respective middle wells with an adjusted P value less than 0.0001. Comparison of the distance moved between the control groups' outer/ middle zone and the treatment groups' outer/ middle zone showed no significance (Figure 7A). Statistical analysis of the total duration spent in a zone showed that the treatment and control groups' outer well was significantly larger than the respective middle well with an adjusted P value less than 0.0001. Comparison of the duration between the control groups' outer/ middle zone and the treatment groups' outer/ middle zone showed no significance (Figure 7B). Analysis of the velocity between the groups showed significance between treatment groups' outer well and middle well with an adjusted P value of 0.0023. There was also significance between the control groups' outer well and the treatment groups' middle well, as well as the control groups' middle well and the tactile groups' outer well (Figure 7C). The heatmaps displayed that the embryo spent a dispersed amount of time in the outer well, but a more concentrated amount of time in one spot of the middle well (Figure 7D).

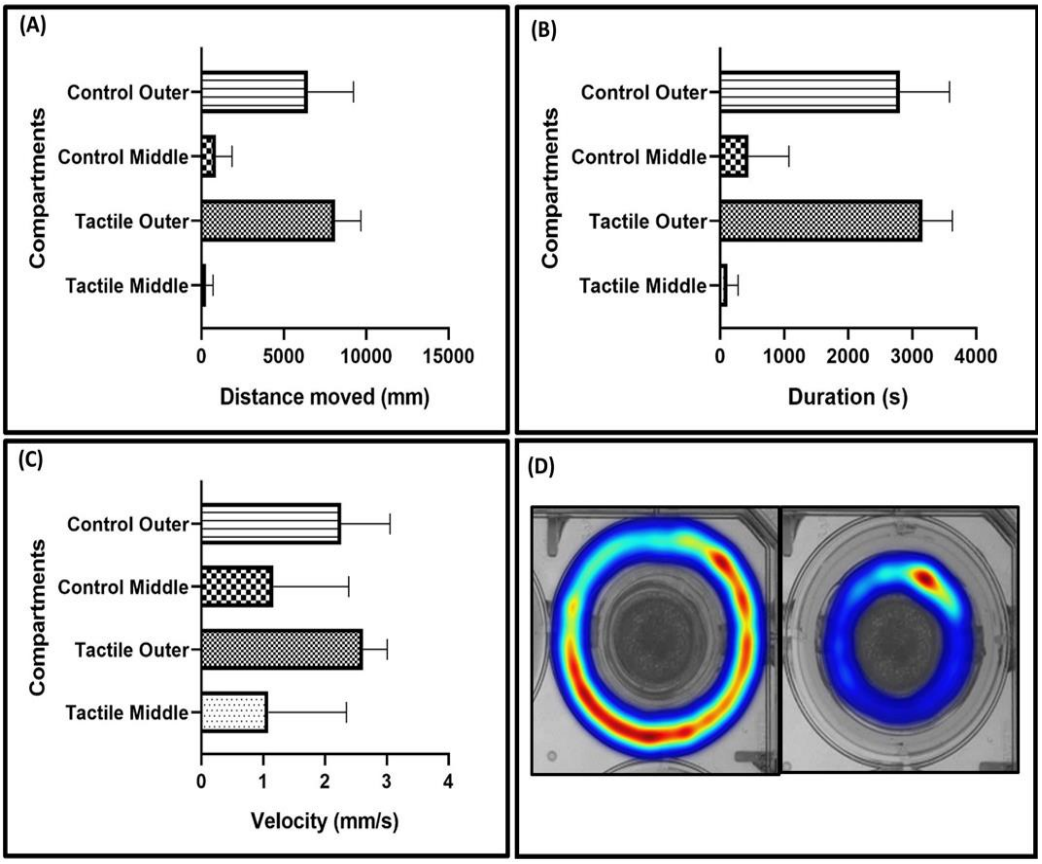

Figure 7. (A) The distance moved (millimeters) around the arena (outer and middle) for the control and treated group. Increased distance moved was seen in the outer well, but there was no significant difference seen between the two groups. (B) The cumulative time (seconds) spent moving around the arena (outer and middle) for the control and treated groups. There was increased time spent in the outer well, but there was no significant difference seen between the two groups. (C) The speed (millimeters per second) around the arena (outer and middle) for the control and treated groups. Increased velocity was seen in the outer well, but there was no significant difference seen between the two groups.

3.3 Social Stimulus

Throughout the White Light Routine, the addition of two embryos in the inner well for the social stimulus did not show a significant change in distance moved, time spent, and velocity between the treatment and control groups. The embryo was much more active in the outer well compared to the inner well in both the treatment and control groups (Figure 8). For distance moved, analysis showed that the control groups' outer well and treatment

groups' outer well were significantly larger than the respective middle wells with an adjusted P value less than 0.0001. There was also significance between the control groups' outer well and the treatment groups' middle well, as well as the control groups' middle well and the tactile groups' outer well (Figure 8A). Statistical analysis of the total duration spent in a zone showed that the treatment and control groups' outer well was significantly larger than the respective middle well with an adjusted P value less than 0.0001. Comparison of the duration between the control groups' outer/ middle zone and the treatment groups' outer/ middle zone showed no significance (Figure 8B). Analysis of the velocity between the groups showed no significance (Figure 8C). However, the heatmaps displayed a very dispersed amount of time in the outer well and middle well. The heat map shows a very equal distribution of time throughout the wells (Figure 8D).

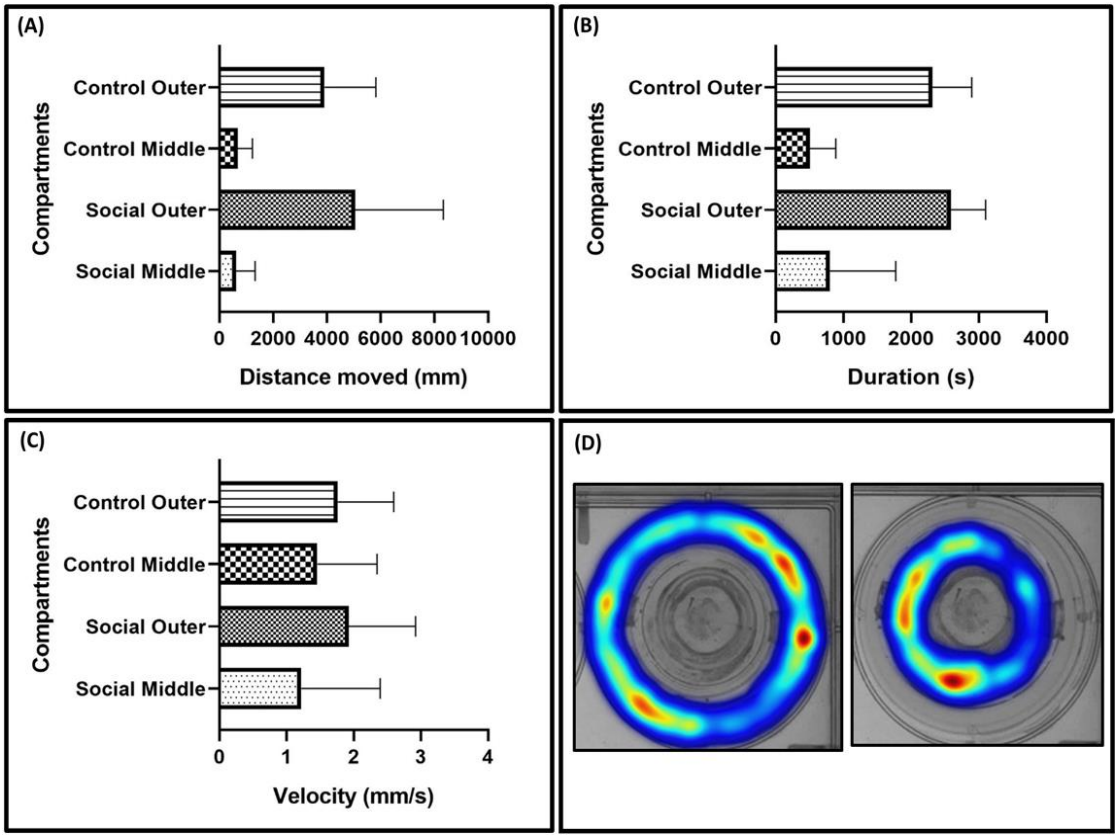

Figure 8. (A) The distance moved (millimeters) around the arena (outer and middle) for the control and treated group. Increased distance moved was seen in the outer well, but there was no significant difference seen between the two groups. (B) The cumulative time (seconds) spent moving around the arena (outer and middle) for the control and treated groups. There was increased time spent in the outer well, but there was no significant difference seen between the two groups. (C) The speed (millimeters per second) around the arena (outer and middle) for the control and treated groups. Increased velocity was seen in the outer well, but there was no significant difference seen between the two groups.

4. Conclusion

Many individuals diagnosed with ASD must experience various symptoms for their entire life. The symptoms of ASD vary from individual to individual; and, as an individual grows and develops, neural plasticity decreases (11). Due to these two factors, treatment for ASD patients can be very difficult; a treatment plan for one individual will never be the same as another, and treatment must be started at a young age for better patient outcomes. A fully

developed behavioral assay that is properly suited to analyze the response of the three stimuli on the zebrafish larvae will allow one to test what can modify the zebrafish's responses to the stimulus and their ability to learn the stimulus. Modifications that help regulate the autistic zebrafish larval behavior and responses can potentially be translated to human autistic patients. This could lead to improved treatment and medications for individuals with ASD.

4.1 Future Studies

Moving forward, it would be beneficial to test if there is a change of results when using autistic zebrafish larvae. If there is a change from the baseline data found in this experiment, then different chemical compounds can be created and/or tested to see if they alter behavior in a positive manner. Using these results, research for more effective pharmaceutical treatment methods for ASD patients can be conducted.

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