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The Effects of D2 Receptor Modulation on Locomotor Development in Danio rerio

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by

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Abstract

This study utilized a novel design to investigate the sensitivity of D2 dopamine receptors to modulating compounds through multiple exposures over early development of zebrafish larvae. Zebrafish were dosed for 30 minutes from 5-8 days post fertilization (dpf) with 16μ/mol of either a D2 antagonist, haloperidol, or a D2 agonist, quinpirole hydrochloride. Two other groups were then dosed with these compounds from 9-12dpf. The effects of D2 receptor modulation were measured by analyzing motor activity on measures of movement distance, frequency, and velocity. Results indicated that larvae dosed with haloperidol on 5dpf had increased activity after the first dosage, but these differences lessened over days 6-8dpf. While conversely, the quinpirole group displayed a decrease in movement activity during trials but in this case, there was a greater deviation from control on trials during 7 and 8dpf. These effects were not observed in subjects dosed from 9-12dpf, which displayed no significant differences from control activity, supporting the hypothesis that those dosed earlier in development would experience greater impacts of D2 modulation. Follow up testing at 16-18dpf did not result in statistically significant differences across treatments, but there were trends on all three measures of lower activity in the quinpirole 5-8dpf group and increased levels of activity in the haloperidol 5-8dpf group. Taken together, these results provide further support for use of *Danio rerio* larval locomotor activity as a measure of D2 receptor modulation as well as evidence for differential impacts on dopaminergic activity dependent upon the period of drug administration.

1. Introduction

1.1. The Dopaminergic System and the D2 Subtype Receptor

The dopaminergic system is essential in the central nervous system's regulation of various bodily functions. These include voluntary movement, reward response, addiction behaviors, mood, cognition, memory, learning, and sleep. Therefore, the balance and functionality of the dopaminergic system have profound impacts on the physical and mental health of an individual (Beaulieu & Gainetdinov, 2011). This system has dispersed neuron populations that operate through a complex network of pathways and receptor subtypes. The present study focuses primarily on the activity of D2 subtype neurons which have their greatest populations in the nigrostriatal pathway consisting of diverse brain regions including the caudate nucleus, putamen, and substantia nigra see Fig.1.
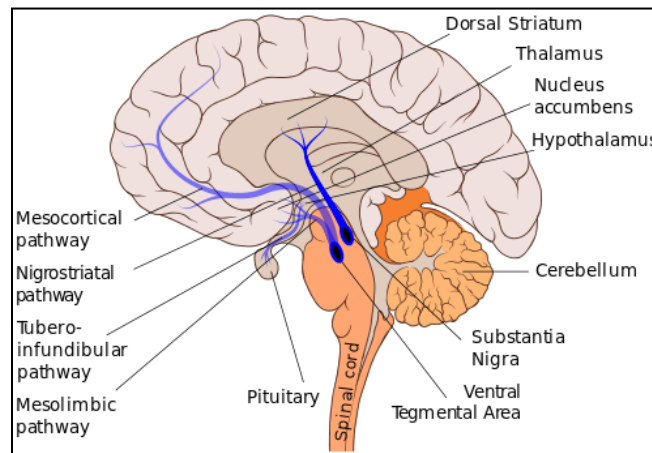
Fig 1. The Dopaminergic System; Regions of Interest: Nigrostriatal pathway, Caudate Nucleus, Putamen Dorsal Striatum, Substantia Nigra, Cerebellum (Lynch & Jaffe, 2007).

The activity and functionality of these pathways is modulated by the exogenous levels of dopamine which circulate between synapses. These exogenous levels which filter throughout the nervous system are regulated by both phasic and tonic modes of transmission (Grace, 1991). The phasic release of dopamine is produced through exocytosis in which a neuron's electrical

gradient is pushed to a critical threshold due to an acute response to external stimuli. This results in the release of dopamine stored within the synaptic vesicle of dopaminergic cells. These dopamine molecules then pass through the synaptic gap to bind to a neighboring neuron which initiates activation and communication of dopaminergic G-protein coupled receptors (GPCRs). Activation of these receptors occurs when a ligand, in this case, dopamine, binds with the extracellular surface of the GPCR causing a conformational change and the subsequent release of G-proteins which have differential actions dependent on cell type and external stimuli. In the case of the D2 dopamine receptor, the focus of the present study, there are linked inhibitory G-proteins which activate through inhibiting the enzyme adenylate cyclase and subsequently, this modulates the activity of several critical cellular pathways such as the P13k-Akt signaling transduction pathway (Beaulieu & Gainetdinov, 2011).

Fig 2. Cross-section of D2 Dopamine G-Protein Coupled Receptor Activation: 1. Ligand binds to the extracellular receptor site of the GPCR (on the left) 2. Leads to conformational change and release of G-protein subunits 3. G-proteins inhibit Adenylate Cyclase allowing downstream activity to initiate (Pescosolido, et al., 2013).

In contrast to the responsive nature of phasic transmission, there is also importantly a tonic transmission of dopamine, a constant "background" release from the frontal cortex into extrace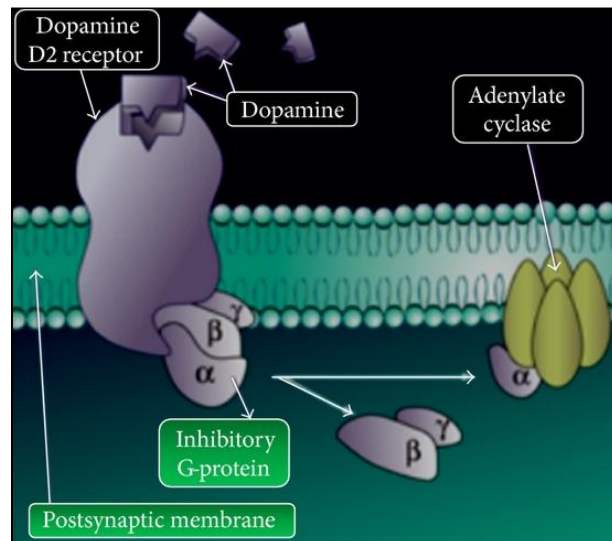llular space between neurons. This tonic transmission has been proposed to regulate the intensity of phasic response and therefore modulation of exogenous dopamine levels would therefore affect the sensitivity and function of dopamine receptors (Grace, 1991). This is of relevance to the present study as previous research has suggested that tonic transmission is regulated through coactivation of the D1 and the D2 receptor (Money & Stanwood, 2013). This would indicate that modulation of D2 receptor activity may have consequences on both D2-specific cellular pathways and the regulation of tonic dopamine transmission to the dopaminergic system as a whole. This may further imply that the activity of the dopaminergic transmission as a whole can be modulated by the presence of external compounds from environmental and pharmacological influences which are able to bind to D2 G-protein coupled receptors. Two methods of action for such compounds are antagonism and agonism. An antagonist compound binds with the postsynaptic membrane of a GPCR in place of the endogenous neurotransmitter without causing the subsequent conformational release of G-protein subunits and thus inhibits the proper activation of the receptor. Conversely, the binding of an agonist to the receptor site does initiate G-protein inhibition, resulting in an increase in activity of the stimulated receptor. The D2 receptor has also been implicated in a variety of critical cellular and neurological processes downstream of activation (Souza et al., 2011; Ek et al., 2012), and several mutations of associated genes have been linked to atypical dopamine receptor signaling (Nerenz 2018). Furthermore, other studies have found an association between this deregulation in dopaminergic activity and the development of various neurological disorders. In order to investigate the potential sensitivity of these receptors to modulation from *in utero* and

neonatal influences, the present study administered *Danio rerio* larvae with either a D2 antagonist, haloperidol or a D2 agonist, quinpirole to observe the downstream locomotor effects.

1.2. The PI3K/Akt Pathway and GABAergic Deregulation

For decades the field of neuroscience has found associations between abnormal dopamine signaling primarily and certain neurological disorders. Recently, a possible explanation of this trend has been discovered as there appears to be a link between the activity of the D2 receptor and the abundance of the protein kinase Akt, a reduction of which is typical in schizophrenic patients (Lai et al., 2006; Beaulieu et al., 2007; Tan et al., 2008). The downstream activity of adenylate cyclase inhibition subsequently initiates the Akt pathway through a complex formation with beta-arrestin and PP2A proteins (Bealieu et al., 2007) as illustrated in Fig. 3. This complex inhibits phosphorylation of Akt and after a series of signaling cascades the Akt protein is translocated to the cytoplasm and nucleus of the cell where it can activate the GSK3 protein leading to a range of cellular functions with critical downstream effects on other aspects of the nervous (Brazil & Hemmings, 2001). These include gene transcription, cell proliferation, and neuronal migration. Therefore, it is postulated that dysregulation of dopamine signaling and subsequently the Akt protein may lead to malformation of neuronal circuitry and functionality. The present study aims to measure this D2 and Akt deregulation through locomotor activity due to previous implications of a connection between D2 modulation and GABAergic transmission, but the level of sensitivity and extent of consequences have yet to be fully elucidated (Souza et al., 2011; Irons et al., 2013; Nabinger et al., 2021).
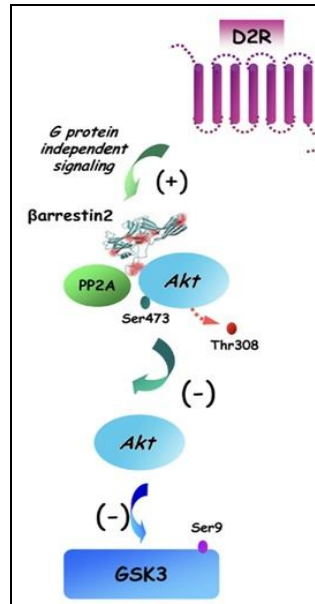
Fig 3. Schematic of Akt Pathway activation: 1. Inhibition of adenylate cyclase initiates further 2. G protein signaling leading to complex formation of βarrestin2 and PP2A 3. Akt pathway activates GSK3 protein leading to regulation of GABAergic system (Beaulieu, et al., 2011).

Utilizing zebrafish larvae as a model, one team observed that through the agonism of D2 receptors the production of Atk will decrease due to a reduction in the precursor compound pAkt. They observed that this suppression of Akt activity subsequently caused a significant decrease in the GABAergic neuron population in the brain and thus led to dysregulation of locomotor behavior (Souza, Romano-Silva, & Tropepe; 2011). The team further observed that a reduction of endogenous dopamine increases movement initiation when the GABAergic system is functional. They then tested whether this relationship was apparent in the opposite direction by reducing the number of GABAergic neurons which, regardless of dopamine levels, caused a significant decrease in initiations of movement. Taken together this suggests that dopamine negatively regulates GABA to control movement behavior and that the GABAergic system is downstream of the dopaminergic in motor regulation. This analysis of zebrafish locomotor

behavior during early development demonstrated evidence of a complex interaction between D2 activation and locomotor activity essential for the current study (Souza, Romano-Silva, & Tropepe; 2011). Taking these previous findings together, the apparent locomotor abnormalities of animal subjects can be traced to the downstream effects of D2 receptor modulation through the Akt pathway; and suggests that differences in motor development are indicative of not only deregulation in the dopaminergic system, but also a decrease in expression of certain genes, and the functionality of cell cycle regulation (Beaulieu et al., 2007; Brown et al.,2012; Lai et al., 2006; Laing et al., 2020).

1.3. History of Antipsychotics and D2-Linked Disorders

The primary area of D2 research, beginning in the 1960s, has been in the effort of pharmaceutical treatment for psychotic conditions, primarily schizophrenia (Bilder et al., 2000; Brown et al., 2012; Kapur et al., 2000). The first antipsychotic medications to be developed, such as haloperidol and loxapine, known as typical antipsychotics, act on the D2 receptor through antagonism resulting in a reduction of D2 activity associated with relief from psychotic symptoms. However, these medications have also displayed a great deal of adverse effects primarily in regards to motor disorders. Due to these apparent risks these drugs are now secondary to atypical antipsychotics in treatment primarily Clozapine, Risperidone, and Olanzapine (Armenteros & Davies, 2006). Unfortunately, these medications can also have severe side effects including neutropenia in which the nervous system has an extreme reduction in white blood cell count (Kumra et al., 2008). In addition, research has suggested that the susceptibility of this condition is far greater in children and varies across race, sex, and age (Maher et al., 2013). Furthermore, the research regarding whether typical or atypical antipsychotics have a greater effect on reducing symptomatology has been inconclusive (Gurevich et al., 2012).

The antipsychotics which are known to cause Parkinsonism, including haloperidol have been shown to bind more tightly to the D2 receptors than atypical antipsychotics which have not been known to cause Parkinsonism. However, this is counterbalanced by shorter-acting relief from psychotic symptoms (Kapur et al., 2000; Seeman & Tallireco, 1998). Therefore, individuals suffering from psychosis are forced to choose between less effective medication or more severe side effects. This is especially important for some patients who only display any symptomatology improvement when using the more tightly bound drugs (de Haan et al., 2003). Research has also displayed a significant correlation between increased D2 receptor occupancy and an increase in clinical improvement. These effects also increased significantly past 65% D2 receptor occupancy (Kapur et al, 2000). This is a serious issue in regards to antipsychotic treatment as other studies have shown that some patients with severe psychosis only experience noticeable relief with D2 occupancy of 60-75% (de Haan et al., 2003). Despite the current understanding of these dangers, the use of both typical and atypical antipsychotics has remained one of few methods in the treatment of both adult and childhood-onset schizophrenia (Armenteros & Davies, 2006; Bartlett, 2013; Gurevich et al., 2012; Sikich et al., 2008).

In recent years, the D2 receptor has also been implicated in a variety of other neurological disorders. For instance, the root of dysfunction in Parkinson's patients has been traced to a reduction in the level of dopaminergic neurons within the striatum mediated by the D2, which leads to the subsequent dysfunction of the thalamus. Further supporting this is evidence that restoration of dopamine activity in the striatum through D1 and D2 activation can alleviate the lack of motor control in Parkinson's patients (DeMaagda & Phillip, 2015). Research has also correlated genes involved in the neurotransmission of dopamine with ADHD (Sachs et al., 2000; Sobel et al., 2010). In particular, a higher level of dopamine transporter gene has been

associated with the presence of ADHD symptomatology. This has been theorized to lead to a reduction of synaptic dopamine levels which subsequently alters neurological morphology. As a possible explanation, studies of the basal ganglia in both animal models and humans have suggested a link between a deficit of dopamine concentration in the basal ganglia and a reduction in number of synapses and a decrease in dendritic spine volume and length (Sobel et al., 2010). Lastly, the D2 receptor is essential for the maintenance of and proper functioning of several frontal cortical areas in the aging brain (Money & Stanwood, 2013). Findings have indicated that a reduction in the distribution of the D2 receptor and an age-related decline in dopamine activity with impairment in frontal and cingulate metabolism leads to difficulties in both motor and cognitive function of elderly subjects including neurodegenerative conditions such as Alzheimer's (Kemppainen et al., 2003; Volkow et al., 1999). This reduction of dopaminergic function has also displayed symptomatology remarkably similar to psychosis that is characteristic of schizophrenia further underlining the D2 receptor as the root of dysfunction (Howard et al., 2000; Jeste et al., 2000). This current body of research on neurological disorders linked to deregulation of D2 receptor activity calls for further investigation of susceptibility to receptor modulation in early development. Further evidence and understanding of this interaction will help to elucidate the progression of numerous neurological disorders. In this effort, the present study analyzed the effects of both haloperidol and quinpirole on larval locomotor activity.

1.4. Haloperidol

Haloperidol, commonly known as Haldol, is a D2 receptor antagonist which was synthesized in 1958 by the Belgian laboratories Janssen, and research of its possible clinical applications began soon thereafter. Within the coming decades, the compound would be selected

as one of the most powerful antipsychotic medications to date and subsequently became the main prescription for treating symptoms of schizophrenia (Granger & Albu, 2015). Following this the compound has been used for the treatment of a variety of general hyperactivity symptoms such as impulsivity, sustaining attention, aggression, mood lability, and poor frustration tolerance (Kudo et al., 1999) and more recently, in the treatment of Alzheimer's and bipolar disorder (Pan et al., 2019; Ashok et al., 2017). The use of this D2 antagonist, however, has been proven to lead directly to the development of movement disorders and therefore, can only be used when the psychosis is severe enough for treatment to be justified (Seeman & Phillip, 2010).

1.5. Quinpirole

Quinpirole hydrochloride is a dopamine agonist that has a high affinity to the D2 subtype. This compound has displayed the highest levels of binding within the striatum, nucleus accumbens, and olfactory tubercles, these areas are linked to the progression of several disorders such as Parkinson's (Koller et al., 1987). As a result, this agonist has also been proposed as a treatment for Parkinson's disease and D2 antagonist induced tardive dyskinesia. This has been further supported by trials indicating the reduction of abnormal movement behavior in a rat model (Choi & Horner, 2021; Koller et al., 1987). However, multiple studies have also displayed the ability of repeated daily postnatal quinpirole exposure in rats to lead to an irreversible D2 receptor supersensitivity (Kostrzewa 1995; Kostrzewa et al., 2003). Hippocampal volumes of brain-derived neurotrophic factor and nerve growth factor were also found to be significantly reduced along with a decrease in the striatum and frontal cortex gene expression within quinpirole dosages subjects (Thacker et al. 2006; Maple et al. 2007). This compound has also been shown to disrupt the Akt pathway and alter locomotor behavior in the zebrafish model in a number of studies (Nabinger et al., 2021; Souza et al., 2011; Oliveri and Levin, 2019).

1.6. Danio rerio as a Developmental Model

The use of animals as neurodevelopmental models has been a cornerstone in the fields of behavioral and neurological research since their founding. Among these models, *Danio rerio*, also known as the zebrafish, has risen to prominence in the fields of pharmacology, toxicology, and neuroscience. This popularity is largely rooted in the practical use of zebrafish larvae is far more simple, replicable, and inexpensive in comparison to other models as a result of large breeding clutches, extremely small size, and low maintenance (Basnet et al., 2019; Kalueff et al., 2014; Nabinger et al., 2021; Stewart et al., 2014). In addition, their life cycle proceeds in an easily predictable manner and they are capable of breeding from maturation until death. A single female can lay hundreds of eggs each week and the embryos are developed into larvae by 72 hours post-fertilization as seen in Fig 4. These embryos are translucent and the organs of the zebrafish develop within one day post fertilization (1dpf) which allows for simple monitoring and manipulation.
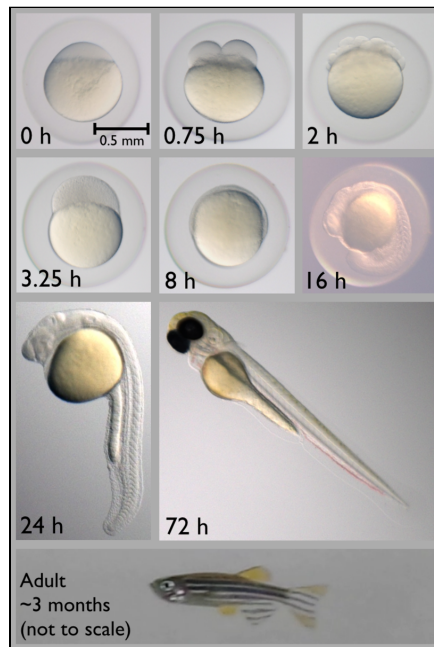
Fig 4. Timelapse of Danio rerio development from embryo to larvae over the course of 72 hours (Hendel, 2004).

Most relevant to the study at hand, this expediency of the central nervous system development presents the zebrafish as a particular model for translation of developmental manipulations (Basnet et al., 2019; Kalueff et al., 2014; Nabinger et al., 2021; Stewart et al., 2014). Furthermore, zebrafish are also known to be sensitive to all major classes of neurotropic drugs, including antipsychotics, mood stabilizers, anxiolytics, antidepressants, and stimulants. Due to this sensitivity and rapid development of the central nervous system the zebrafish is an excellent model for investigating early life drug exposure and the effects this has on subsequent development and behavior. Prior studies have also observed a variety of developmental phenotypes both social and behavioral, believed to be linked to the deregulation of dopaminergic transmission (Dreosti, et al., 2015, Stewart, et al., 2014).

For the present study, this model was selected due to the conservation of relevant cortical regions and the translatability of neuronal manipulation with humans. In 2001 researchers successfully analyzed the entire genome and located at least one orthologue, a string of homologous DNA deriving from a common ancestor, in zebrafish for 71.4% of human genes as well as at least one human orthologue for 69% of zebrafish genes (Howe.K, et al., 2001). This research has suggested that despite the apparent evolutionary distance between teleosts and mammals there is a great deal of homology in the functionality of key brain regions including the olfactory bulb, cerebellum, spinal cord and habenula which regulates the release of serotonin and dopamine in the nervous system (Aizawa et al., 2010; Aizawa et al., 2011). Comparisons of these structures in both central nervous systems can be seen in Fig. 5.
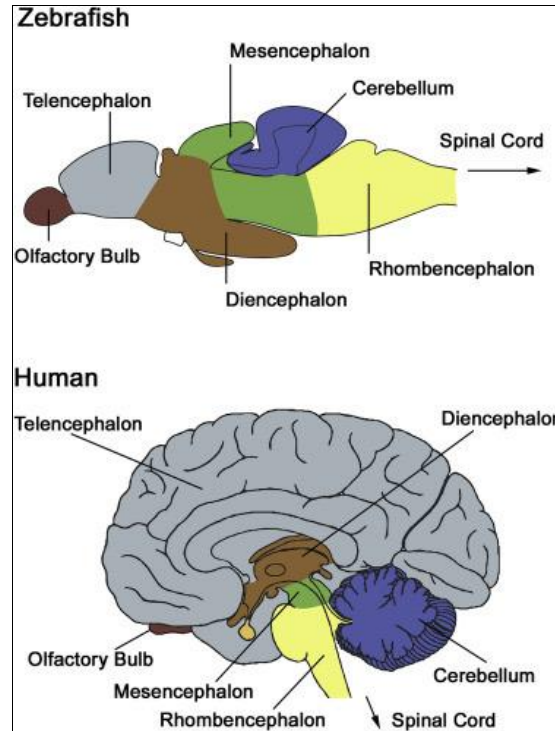
Fig 5. Comparison of the evolutionarily conserved regions of the dopaminergic system between the zebrafish and human central nervous systems; Key area of interest: telencephalon and diencephalon (Bartel, et al., 2020).

Studies have also been conducted to discover the genomic sequence specifically for segments pertaining to D2 receptor genes in the zebrafish model. One team identified several localized areas which coded for three different D2 subtypes, two of which occurred on the analogous chromosome in the human genome and they found 70% overall homology of the receptor between the two species (Bohmler, et al., 2004; Ek 2012). In addition, the neuronal pathways which are responsible for locomotion in *Danio rerio*, the reticulospinal neurons, and descending vestibulospinal projections are evolutionarily conserved among vertebrates (Basnet et al., 2019; Du et al., 2016; Kunst et al., 2019). The dorsal and ventral nuclei of the teleostean ventral telencephalic area are also believed to be analogous to the striatum and septum of other vertebrates (Wulliman & Rink, 2002). Lastly, the development of the dopaminergic system in

zebrafish begins around 15-18hpf and the entire system including neuronal projections are fully established by the end of 4dpf (Boehmler et al., 2004; Stickney et al., 2014). Therefore, both the analogous nature and rapid growth of the dopaminergic system in zebrafish present it as an extremely useful neurodevelopmental model for the present investigation of D2 receptor modulation.

1.7. Rationale for Study

While the sensitivity of the dopaminergic system has become clearer, concerns about early life damage and subsequent outcomes have become increasingly important because this system is linked to the onset of several prevalent neurological disorders. Among the greatest risks for this type of early developmental damage in humans is intrauterine drug exposure (Bell & Lau, 1995; Julvez & Grandjean, 2009; Kwong & Ryan, 1997). However, the majority of studies investigating this issue have focused on drug abuse of illegal substances while little has been done in comparison to elucidate the interactions of prescribed pharmaceuticals (Bjørling-Poulsen, 2008; Ross et al., 2015). For instance, the influence of cocaine *in utero* has been associated with both increase in premature labor and a variety of developmental delays such as microcephaly, cardiac and genitourinary abnormalities, and central nervous system stroke (Young & Phillips, 1992). This is noteworthy considering the main region of effect under the influence of cocaine is in dopaminergic transmission which underlies the mechanisms of action in pharmaceuticals including antipsychotic medication which is used in treating a variety of disorders. One of few meta-analyses on the neurodevelopmental consequences linked to intrauterine exposure of antipsychotics found significant effects of dopaminergic dysfunction in the case of animal models, but less substantial evidence in humans (Poels et al., 2018). These developmental issues in animal studies are exemplified through decreased brain weight, lack of

certain neurocircuit communications, as well as behavioral changes characterized by avoidance and anxiety-like phenotypes (Basnet et al., 2019, Du et al., 2016, Souza & Tropepe, 2011). Despite less evidence of effects in humans, these findings remain relevant as these changes in animal models occur along the same evolutionarily conserved pathways as human neurological disorders such as Parkinson's disease and schizophrenia (Howe.K, et al., 2001; Aizawa et al., 2010; Aizawa et al., 2011). Therefore, given the current lack of understanding in the field, it is critical to gain greater knowledge of the long-term effects of D2 receptor modulation. The present study utilized the zebrafish as a neurodevelopmental model in order to investigate the early developmental effects of this D2 modulation through administration of either a D2 antagonist, haloperidol, or a D2 agonist, quinpirole.

1.8. Hypotheses

Given the current literature on D2 modulation and subsequent downstream effects, the present study had three separate hypotheses to investigate different aspects of developmental effects. Firstly, both groups of larvae dosed with haloperidol from 5-8dpf and 9-12dpf would exhibit increased activity on measures of movement distance, movement frequency, and average velocity from control subjects while groups dosed with quinpirole would exhibit decreased activity from the control. Secondly, larvae dosed from 5-8dpf would display more significant differences from the control over the period of testing than their counterparts dosed from 9-12dpf. And lastly, during follow up trials from 16-20dpf groups dosed from dpf5-dpf8 would exhibit more significant variations in movement activity from the control than their counterparts that were dosed from 9dpf-12dpf.

2. Methods

2.1. Study Design and Procedure

Beginning on 5dpf two groups of 15 zebrafish larvae were dosed for half an hour with 16μ/mol D2 modulating compounds, group one with haloperidol, a D2 antagonist, and group two with quinpirole, a D2 agonist. After this half-hour dosing period, four subjects from each group along with four control subjects were placed into individual arenas of a 3x4 well plate (Appendix. B.). These well plates were then tracked and movement activity was analyzed over the course of four ten-minute trials. Afterward all subjects were returned to their original Petri dishes with EW solution. This procedure was then repeated over the next three days from 6-8dpf. Following 8dpf these two groups were no longer administered. On 9dpf two new groups of larvae that had not previously been dosed underwent an analogous procedure to those dosed from 5-8dpf. These groups were then dosed over 10-12dpf. This study design provided these four experimental groups to investigate differential motor behaviors modulated by both the drug method of action and period of drug administration. Following final drug administration on 12dpf all groups were cared for in preparation for follow up testing from 16-20dpf. However, in the present study, there was an unexpectedly high death rate in the haloperidol 5-8dpf group, and as a result data collection only occurred on 16 and 17dpf. These follow up tests were identical to previous data collection with the exception of no further drug dosing. The drug administration and euthanasia protocol used in this design was approved by the Bard College IACUC.

Group 1: Haloperidol 5-8dpf	**Group 3:** Haloperidol 9-12dpf
Group 2: Quinpirole 5-8dpf	**Group 4:** Quinpirole 9-12dpf

Fig. 6 Table laying out the 2x2 design of the four treatment groups utilized in the present study

2.2. Fish Husbandry

Approximately 100 zebrafish embryos were produced from TU wild-type zebrafish and remained in a controlled environment until 4dpf wherein they were transferred to the testing site and separated into six groups of 15 subjects (4 experimental groups and 2 control groups). Here they were housed inside an incubator set at 28℃. For the first three days, the larvae received nutrients from an egg water (EW) solution designed for their early development. This EW is a solution of deionized water with concentrations of 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 10-5 % Methylene Blue. Proper concentrations were gained by diluting a stock solution. Beginning on 4dpf, larvae were cared for twice a day first at 9:00 am and 5:00 pm, each petri dish was cleaned, EW solution was replaced and the larvae were given a small amount of micron fish food.

2.3. Drug Administration

Starting from 5dpf both the 5dpf-8dpf groups were dosed for half an hour (from approximately 3:30-4:00 pm) with 16μ/mol of haloperidol and quinpirole respectively which were ordered from Sigma-Aldrich Inc. (ST. Louis, MO). Dosing of 16μ/mol of haloperidol and quinpirole was based on the findings by Irons et al., 2013 that displayed the most significant movement results at this dose as opposed to higher or lower concentrations. The drug stock solution of haloperidol was created by first mixing 8mL of dimethyl sulfoxide into 40 mL of EW and dissolving 12.8mg of haloperidol into the mixture. For dosing 1mL of this stock was added to 49mL of EW and subjects were then placed in the solution for 30 minutes. The duration of dosage was determined by previous studies and results from Pilot Study 1 (see Appendix. T.). The drug stock solution for quinpirole was made by dissolving 6.8mg of the drug into 16mL of EW. For dosing 500 micro/mol of stock was placed into 49.5 mL of EW to gain the proper

concentration. This procedure was repeated from days 5 through 8 after which the 9dpf-12dpf groups underwent an analogous procedure for the next 4 days. Following the final drug dosing on 12dpf, larvae were maintained and cared for until 16dpf wherein locomotor tests resumed. Larvae's locomotor activity was analyzed once again from 16-18dpf the previous procedure but without further drug administration.

2.4. Locomotor Data Collection

For each test 12 subjects were placed in a 4x3 well-plate, 4 controls, 4 treated with haloperidol, and 4 treated with quinpirole. The arenas were placed inside of the DanioVision™ lightbox (Noldus Information Technology, Wageningen, the Netherlands) and their movement was tracked and analyzed using the EthoVision XT® tracking software (Noldus Information Technology, Wageningen, the Netherlands). Inside the lightbox, an infrared camera selects for each of the 12 subjects and tracks their locomotor activity across 40 minutes segmented into four 10-minute trials. The EthoVision XT® software then receives this input and records data every few milliseconds. Following data collection, each trial was exported to Microsoft Excel where all the data gathered over the trial for each subject is presented in a spreadsheet. This raw movement data was then organized in order to calculate the movement behavior of each subject per 10-minute trial, from this data total distance moved in cm, average velocity, and movement frequency were calculated for each subject across all conditions. Decisions on the duration of trials and measurements of locomotor activity were based on findings from pilot studies 2 and 3 (See Appendix. U. & V.).

2.5. Statistical Analysis

Statistical comparisons were performed through a series of two-way repeated measures ANOVAs on measurements of total movement distance in cm, velocity cm/s, and movement frequency (number of total movement initiations over trial) through Jamovi statistical software (Jamovi Project, Sydney Australia). This analysis implemented a two-way 3x4 repeated measures ANOVA in order to compare differences among groups on both the between-subjects factor of "Treatment" with the factors "Control, Haloperidol, and Quinpirole" and the within-subjects factor of "Trial" with factors of "Trial 1, Trial 2, Trial 3 and Trial 4". Separate ANOVAs of this design were performed for movement measures of distance, frequency, and velocity for each day of testing. These tests represent four trials with four subjects in each of the three treatments. However, due to the nature of the zebrafish model, it is difficult to distinguish between individual subjects and so repeated measures analysis over multiple days of testing was not viable for this experiment.

3. Results

3.1. Trials From 5-8dpf

5dpf Distance

Results of trials recorded on 5dpf directly following the first drug administration provided evidence of D2 antagonism leading to an increase in the movement behavior of larvae on measures of total distance, movement frequency, and average velocity. A two-way repeated measures ANOVA of the data recorded on 5dpf presented both a main effect of trial on 5dpf $F_{(3, 27)} = 67.02$, p= <.001 along with an interaction of trial and treatment $F_{(6,27)} = 4.09$, p= .005.
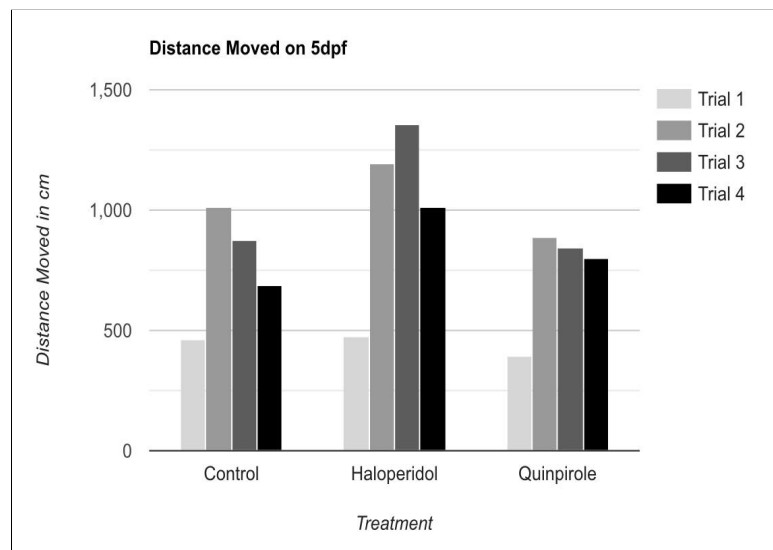
Fig. 7 Distance moved on trial 1 had little variance, but during trials 2 and 3, the haloperidol drastically increased in total distance in a significantly different pattern from both control and quinpirole subjects. Though activity lessened on trial 4 it was still higher than both other groups; SEs included in Appendix. C.

A post hoc analysis comparing differences between trials displayed significant effects of higher scores during later trials. This can be seen in comparisons of results on Trial 1(M=112, SD=28.7) with Trial 2 (M=258, SD=59.2)$t(9) = -13.584$, p= <.001, on Trial 1 (M=112, SD=28.7) with Trial 3 (M=256, SD=79.5)$t(9) = -11.199$, p= <.001 and again between Trial 1 (M=112, SD=28.7) and Trial 4 (M=209, SD=60.8)$t(9) = -6.722$, p= <.001. Further analysis also revealed

that this main effect of trial was largely due to the interaction with treatment and in particular the activity of the haloperidol dosed larvae. Post hoc tests comparing scores of haloperidol dosed subjects between trials confirmed this, firstly activity on Hal Trial 1(M=119, SD=36.2) with Hal Trial 2 (M=298, SD=85.5) $t(9) = -9.06$, p= <.001, on Hal Trial 1(M=119, SD=36.2) with Hal Trial 3(M=339, SD=44.1) $t(9) = -9.79$, p= <.001, and on Hal Trial 1(M=119, SD=36.2) with Hal Trial 4(M=253, SD=90.6)$t(9) = -5.33$, p= .012, all provided significant results. Neither of the other groups displayed any significant post hoc effects. This confirms that the control and quinpirole treated larvae displayed little variance across the four trials while the haloperidol group had significant increases over the course of both Trial 2 and 3; this is illustrated in Fig. 7.

5dpf Frequency

The results for 5dpf on the measure of movement frequency provided further evidence of the D2 altering locomotor activity. Analysis indicated a main effect of trial $F(3,27) = 5.77$, p = .003, an interaction of trial and treatment $F(6,27) = 4.87$, p= .002, and a between subjects main effect of treatment $F(2,9) = 5.7$, p= .0025. Post hoc testing further confirmed that movement of the haloperidol group was significantly different from both control and quinpirole dosed groups Hal (M=123, SD= 112.75) and control (M=13.5, SD=15.04)$t(9) = 2.91$, p= .042, Hal (M=123, SD=112.75) and Quin (M=12.5, SD=12.88) $t(9) = -2.93$, p= .04. Tests of the interaction between trial and treatment also revealed a significant result of changes in the haloperidol dosed group across trials Hal Trial 1(M=34.5, SD=27.4) and Hal Trial 2(M=148, SD=103)$t(9) =-4.29$, p= .046, Hal Trial 1(M=34.5, SD=27.4) and Hal Trial 3 (M=211, SD=141) $t(9) = -5.148$, p= .016. Thus, confirming that changes in movement frequency occurred in a similar pattern across trials as the results on distance. All three groups displayed similar results on trial 1 followed by haloperidol subjects sharply increasing in movement activity during trials 2 and 3.
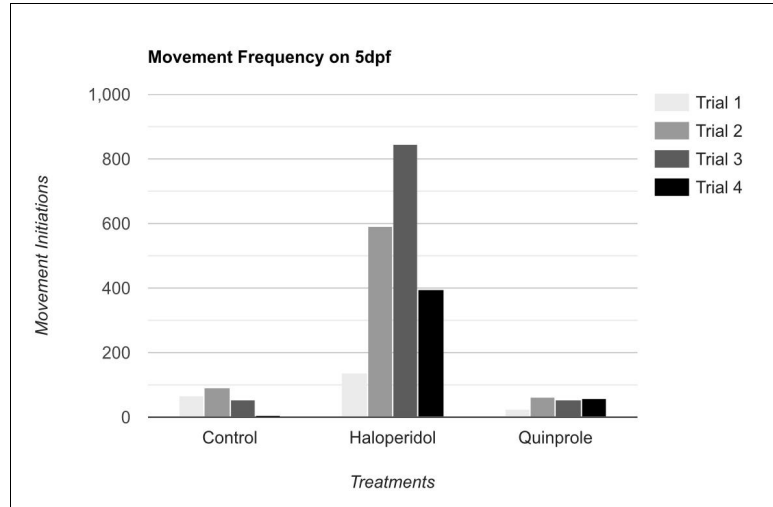
Fig 8. Frequency of movement initiations across trials recorded on 5dpf display a significant difference in activity of the haloperidol dosed larvae with both the control and quinpirole dosed subjects. Similarly to scores of total distance, the frequency increased significantly during Trial 2 and 3; SEs included in Appendix. D.

5dpf Velocity

Similarly to measures of distance and frequency the results of average velocity on 5dpf displayed both a main effect of trial on 5dpf $F(3,27) = 5.55$, p= .004 and an interaction of trial with treatment $F(6,27) = 2.79$, p= .031.
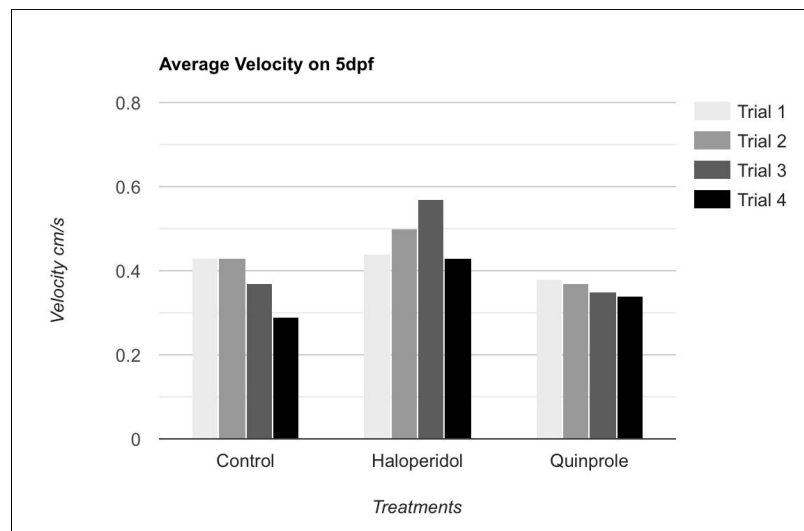
Fig 9. Results of the average velocity across trials recorded on 5dpf similarly displayed an increase of activity in haloperidol-dosed subjects during Trials 2 and 3; SEs in Appendix. E.

Post-hoc analysis revealed that the increases in velocity of the haloperidol group were not statistically significant from the control, but it is important to note that the pattern is similar to the other two measures as illustrated in Fig. 9.

6-7dpf Distance

On days 6 and 7 there were only main effects of trial and no significant interactions , 6dpf $F(3,27)= 3.03$, p= .046, and 7dpf $F(3,27) = 4.841$, p= .008. While there were no significant interactions or effects of treatment there were trends suggesting a decreased activity in quinpirole subjects during trials on 6 and 7dpf in similar patterns to frequency as seen in Fig. 10.

6-8dpf Frequency

Similarly to the measure of distance, there were no significant effects on day 6 and on day 7 there was only a main effect on trial $F(2,9) = 4.47$, p= .01. After removing three outlier scores from control scores on 7dpf there was a significant between-subjects effect of treatment $F(2,9) = 5.73$, p= .029 and a post hoc showed a significant difference between control (M= 214 , SD= 176.75) and quinpirole treated larvae (M=16.37, SD= 12.65) $t(7) = 3. 837$, p= .015, as well as between haloperidol (M= 202, SD= 146) and quinpirole (M=16.37, SD= 12.65) $t(7) = 3.5$, p= .024, treated subjects. These results are illustrated in Fig. 10. Together these findings from 6-8dpf provide evidence that quinpirole-dosed subjects exhibited lower activity due to D2 modulation during 6 and 7dpf. In addition, considering the significance of haloperidol activity in 5dpf trials the lack of such effects on subsequent testing may be indicative of increased resistance to antagonism over multiple drug administrations.
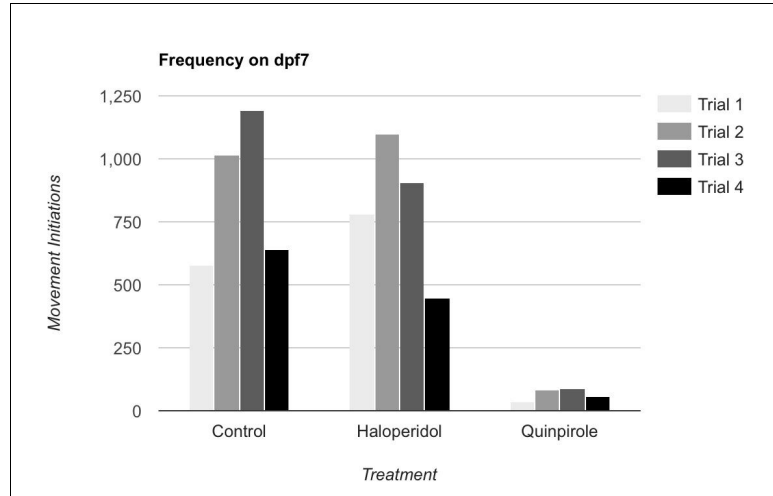
Fig. 10 Results of movement frequency on 7dpf display a significantly lower amount of movement initiations in comparison to both control and haloperidol-dosed subjects. Outliers included in figure; SEs in Appendix. F.

There were also no significant results of velocity over 6-8dpf displayed no significant effects and there were none on any measure during trials on 8dpf.

3.2. Trials From 9-12dpf

9dpf Distance

Larvae dosed for the first time on 9dpf displayed a main effect for trial $F(3,27) = 18.422$, $p= < .001$, but in this case, had no interaction with treatment, and all subjects displayed similar movement patterns and increases across trials as seen in Fig. 11. While there were main effects of trial, post hoc analysis revealed no relationship with treatment. This differential response of haloperidol treated subjects from the 5-8dpf counterparts supports the present study's hypothesis for greater sensitivity to modulation in the larvae dosed from 5-8dpf.
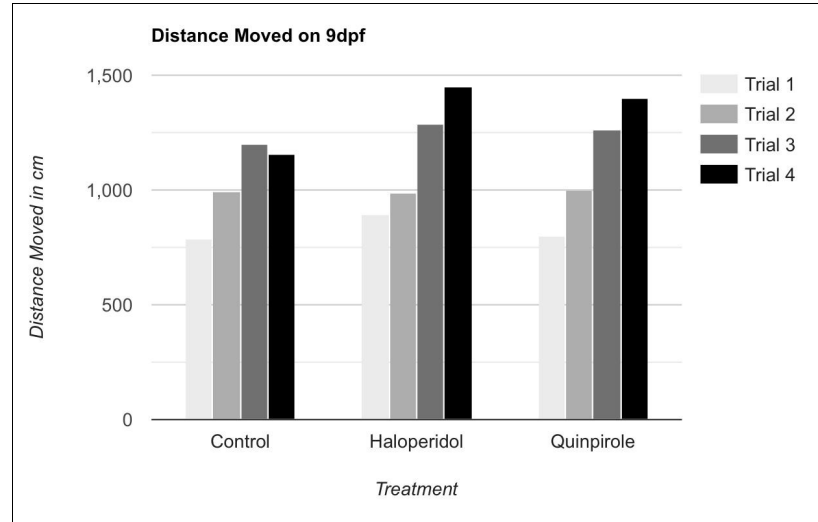
Fig 11. Results of the total distance moved across all trials displayed no significant differences between groups and a similar pattern of increasing activity as trials progressed; SEs in Appendix. G.

9dpf Frequency

Larvae dosed on 9dpf displayed a main effect of trial on the measures of frequency $F_{(3,27)} = 18.173$, p= < .001, however, there was no interaction with treatment. In conjunction with results of distance moved this suggests that larvae treated on 9dpf exhibited locomotor activity similar to control subjects. In addition, there were no significant effects of velocity on 9dpf. While larvae dosed with haloperidol on 5dpf exhibited significant variation on all three measures their counterparts first dosed on 9dpf had movement patterns which had no significant variations from control subjects.

10-12dpf Activity

On days 10-12dpf the control groups exhibited higher activity on all three measures while both treated groups displayed similar patterns. There were no significant effects on movement behavior related to treatment until 12dpf where Interaction of trial and treatment on all three measurements, distance: $F_{(6,27)} = 4.74$, p= .018, frequency: $F_{(6,27)} = 2.87$, p= .027, and velocity $F_{(6,27)} = 4.91$, p= .002. However, unlike the interactions observed on 5dpf this

interaction resulted from the control group scoring significantly higher than both drug-dosed groups. Overall, there were more significant variations in the 5-8dpf groups especially in regard to the haloperidol 5-8dpf group on the first day of dosing which may indicate a higher level of D2 receptor sensitivity during earlier stages of development.

3.3. Trials From 16-17dpf

Data collected on both 16 and 17dpf did not provide significant differences between subjects on any of the three measures. However, trends in this data do provide some evidence of differential patterns in motor activity. The most apparent trend was a lower level of activity in larvae dosed with quinpirole on 5-8dpf in comparison with all other subjects across all three measures. Though less consistent there also appears to be a trend of higher activity in larvae dosed with haloperidol during 5-8dpf. In addition, both 9-12dpf groups continued to display lower levels of activity compared to control subjects just as in trials conducted from 10-12dpf suggested. While results were non-significant these trends were in line with the hypothesis that differential long-term effects are dependent upon age of dosing. Figures representing data collected from these trials are included in the appendix of this study.

4. Discussion

4.1. General Discussion

On 5dpf, the first day of drug administration, larvae in the haloperidol treated group displayed significant increases in movement activity of larvae dosed with haloperidol over the course of the four trials while the control and the quinpirole group remained consistent. While all three groups displayed similar results on trial 1 followed by haloperidol sharply increasing. This relationship was observed on all measures and most significantly on movement frequency. Furthermore, results displayed increases in distance and frequency occurring in conjunction with one another throughout trials which further supports evidence of D2 modulation. This is also supported by the scores on velocity which display an increase in average velocity in a similar pattern. Thus, there is significant evidence that on 5dpf the haloperidol treated group's behavior was affected by drug treatment. Additionally, in consideration of previous evidence that D2 modulation disrupts the dopamine system's regulation of GABAergic activity, it is postulated that more distance traveled in larvae dosed with haloperidol is indicative of the increase in movement initiations themselves and therefore could suggest Parkinson-like motor deregulation.

The effects of D2 modulation over the course of 6-8dpf were less apparent, nonetheless, some interesting trends were observed. Firstly, on 6dpf though not significant there was a higher level of activity of the haloperidol dosed group on measures of distance moved and frequency. Furthermore, there was a significant decrease in movement initiations of quinpirole dosed subjects on 6dpf. Then on 7dpf the pattern of results changed to reflect similar movement activity in control subjects as those doesd with haloperidol while the quinpirole group displayed lower movement distance and frequency. On 8dpf yet another differential pattern emerged in which both the haloperidol and quinpirole groups displayed less activity than the control. These

differences may be due to the individual differences in subjects tested across days of dosing. Though there were no significant effects of treatment from 6-8dpf, the distance moved across these four days of testing does display a consistent trend of higher activity in the group dosed with haloperidol. In addition, larvae in the quinpirole group exhibited little differences from control at the beginning of dosing, but appear to decrease in activity in the last two tests on 7 and 8dpf. Thus, the results of this study support the hypothesis that D2 modulation would have inverse effects dependent on the type of D2 binding compound administered. Importantly, these results display significant differences on 5dpf with non-significant trends over the next three days of dosing. This may suggest that the dopamine system at 5dpf has either become stable enough to resist changes in signaling or has adapted to administration of these specific compounds. Alternatively, this could suggest that the dosage used in this procedure was minimal enough for the system to adapt to the modulation.

While larvae dosed with haloperidol on 5dpf exhibited significant increases in activity there was no such effect in those first dosed on 9dpf. Rather, the larvae dosed on 9dpf exhibited similar movement activity to control subjects on all measures. This suggests that at 9dpf modulation of D2 receptors did not alter the dopamine system significantly enough to affect motor behavior. During subsequent tests from 10-12dpf treated groups displayed some differences from the control group, but in a different pattern than the 5-8dpf subjects. In this case, both haloperidol and quinpirole groups displayed gradually increasing differences from control, but not from one another. Essentially, the larvae dosed from 9-12dpf did not exhibit significant differences from the control, and differences that did arise followed no apparent pattern. In sum, the data recorded from these four experimental groups provided support for the hypothesis that those dosed during the earlier period of 5-8dpf would experience more

significant effects of dosage, but did not elucidate whether this had long-term effects on motor behavior.

To test this final hypothesis, there was a planned third round of data collection of all subjects from 16-20dpf. However, data was only collected on days 16 and 17, instead of the planned testing until 20dpf, due to an unexpectedly high death rate of the 5-8dpf haloperidol group. On 18dpf only 3 subjects in the haloperidol 5-8dpf group remained so testing was halted early. Although data collection ended prematurely, trials recorded on 16 and 17dpf did display some interesting differences in locomotor activity, particularly in the case of groups dosed from 5-8dpf. There were trends of the haloperidol 5-8 group displaying a slightly higher level of activity than all other treated subjects on each measure of activity on both 16 and 17dpf. Alternatively, the quinpirole 5-8 group displayed the lowest scores on movement distance and velocity across both days. Along with low scores in frequency that were made non-significant by an unexpected low movement frequency in the haloperidol 9-12dpf group. Though these effects are non-significant they serve as a preliminary glimpse at possible long-term differences in motor activity of larvae dosed with D2 modulating compounds.

Taken together, the results of this study provided support for two central hypotheses firstly, that D2 antagonism and subsequent GABAergic deregulation led to increased activity on all measures of movement activity, and secondly, that D2 modulation had differential effects on larvae locomotor behavior dependent on the period of dosage. Results of trials from 5-8dpf found that haloperidol dosed larvae increased in activity significantly during the first day of dosing while quinpirole dosed subjects displayed some significant decreases in activity on 7 and 8dpf. In addition, differences in behavioral effects of drug manipulation by the period of dosage were observed by comparing the acute effects of D2 antagonism on larvae first dosed on 5dpf as

opposed to those first dosed on 9dpf. While the third hypothesis remains open for future investigation, the present findings support the continued use of *Danio rerio* locomotor activity as a measurement of sensitivity to D2 receptor modulation throughout early development.

4.2. Limitations and Future Directions

Though this novel study provided promising results there were some key limitations to the design that future research can build on. Firstly, the time-sensitive manner of the procedure created several constraints to the methodology of the study. For instance, the design was meant for a larger number of test subjects, however, breeding clutch sizes are unpredictable and this led to the use of smaller treatment groups than desired. This is a significant limitation not only due to lowering the sample size of statistical analyses but also because smaller groups of larvae can experience a significant die-off within the first 20 days post fertilization that this experiment examined. Such a die off occurred in the group dosed with haloperidol from 5-8dpf and while it is possible that this high death rate is related to D2 modulation such speculation is outside the confines of the present study. The other major limitation of the present study is the identification of individual larvae. As these larvae are essentially identical to the naked eye there was no reasonable way to assess which individual larvae were selected for testing on a particular day. As a result, the repeated measures ANOVAs were limited to analyzing comparisons of groups over the four trials performed on a single day of testing and thus there was a much smaller sample size for statistical analysis.

A starting point for the improvement of this design would be the use of larger testing arenas in order to simultaneously analyze a larger sample size. This could then be made more significant by ensuring that the same individual larvae were analyzed in the same arenas over multiple days of dosage. Which would allow for a more meaningful comparison of locomotor

activity over the course of development. In addition, future investigations should assess the effects of D2 modulation on earlier days of larval development to assess impacts on the dopaminergic system as *in utero* influences of D2 modulation in humans may well occur prior to the full development of this system. Due to the wide ranging-downstream effects of the D2 receptor, this future research may be instrumental in the treatment of conditions including but not limited to: motor dysfunction, psychosis, and delusion related disorders, neurodegenerative disorders such as Alzheimer's, and even some forms of cancer (Seeman & Phillip, 2010; Weissenrieder et al., 2019).

4.3. Conclusions

While previous studies have confirmed that a relationship between D2 modulation and locomotor activity exists, the level of system sensitivity and longevity of consequences has yet to be fully elucidated (Souza et al., 2011; Irons et al., 2013). Many of these studies utilized high dosages of medications, exceeding 80% occupancy of the D2-receptor sites which cause severe side effects (Kapur et al., 2000; Du et al., 2016). This prolonged level of exposure has allowed researchers to reliably determine that behavioral differences which arise can assuredly be linked to the drug treatment. However, this prolonged exposure is not readily translatable to what human infants may experience from intrauterine or neonatal influences. In addition, research has displayed that even a single acute administration of D2 modulating compounds are able to dysregulate D2 activity and cause a litany of downstream effects (Bealieu et al., 2007; Souza, Romano-Silva, & Tropepe; 2011). For these reasons, this study aimed to assess how repeated short exposures of D2 modulating compounds can affect the locomotor behavior of the zebrafish to better understand how sensitive these receptors are during the early stages of neurodevelopment. In this effort, the study provided potential evidence of differential effects due

to D2 modulation dependent upon both method of action and period of dosage. Most significantly, the results of larvae dosed from 5-8dpf exhibiting greater variation from their counterparts dosed from 9-12dpf provides support for the future implementation and improvement of similar study paradigms to further elucidate possible critical periods for the neurodevelopmental consequences of dopaminergic modulation. Future investigations in this area of research should also focus on the environmental and pharmacological factors which act on the D2 receptor as this will be instrumental; in not only understanding the progression and root of D2-linked disorders but also in promoting the development of new and more beneficial ligands.

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Appendices

Appendix. A. Study Apparatus

Noldus DanioVision Lightbox

EthoVision XT software during trial acquisition

Appendix. B. Testing Arenas
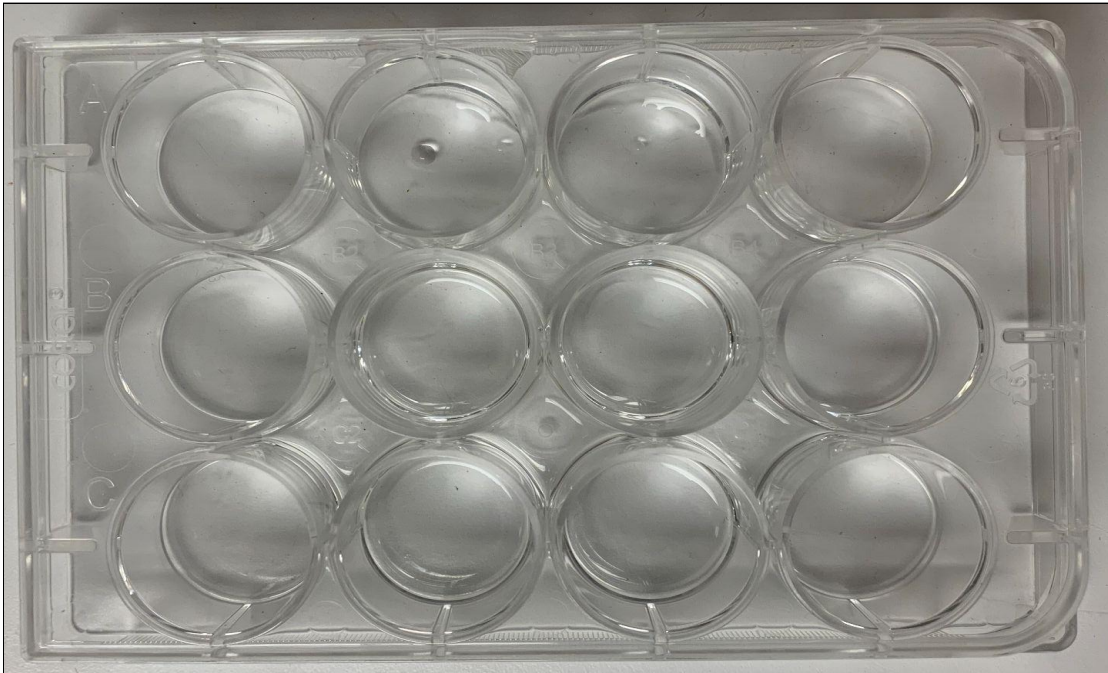
Photo of 3x4 arena well-plate

Control	Control	Control	Control
Haloperidol	Haloperidol	Haloperidol	Haloperidol
Quinpirole	Quinpirole	Quinpirole	Quinpirole

Placement of subjects in 3x4 arenas for data collection

Appendix. C. Distance Moved on 5dpf in cm (Fig. 7)

Treatment and Trial	N	M	SD	SE
Control Trial 1	4	117	23.3	11.7
Control Trial 2	4	253	21.7	10.9
Control Trial 3	4	220	39.8	19.9
Control Trial 4	4	172	16.6	8.32
Haloperidol Trial 1	4	119	36.2	18.1
Haloperidol Trial 2	4	298	85.5	42.8
Haloperidol Trial 3	4	339	88.3	44.1
Haloperidol Trial 4	4	253	90.6	45.3
Quinpirole Trial 1	4	98.9	28.8	14.4
Quinpirole Trial 2	4	222	33.6	16.8
Quinpirole Trial 3	4	211	13.4	6.72
Quinpirole Trial 4	4	201	25.7	12.9

Appendix. D. Movement Frequency on 5dpf (Fig. 8)

Treatment and Trial	N	M	SD	SE
Control Trial 1	4	16.5	20.8	10.4
Control Trial 2	4	22.5	17.6	8.78
Control Trial 3	4	13.8	11.9	5.94
Control Trial 4	4	1.25	2.5	1.25
Haloperidol Trial 1	4	34.5	27.4	13.7
Haloperidol Trial 2	4	148	103	51.5
Haloperidol Trial 3	4	211	141	70.7
Haloperidol Trial 4	4	99	119	59.4
Quinpirole Trial 1	4	5.75	6.4	3.2
Quinpirole Trial 2	4	15.8	15.2	7.6
Quinpirole Trial 3	4	13.8	9.32	4.66
Quinpirole Trial 4	4	14.8	21	10.5

Appendix. E. Average Velocity on 5dpf in cm/s (Fig. 9)

Treatment and Trial	N	M	SD	SE
Control Trial 1	4	.431	.087	.043
Control Trial 2	4	.426	.037	.018
Control Trial 3	4	.367	.0670	.033
Control Trial 4	4	.290	.027	.014
Haloperidol Trial 1	4	.442	.134	.066
Haloperidol Trial 2	4	.503	.144	.072
Haloperidol Trial 3	4	.567	.147	.073
Haloperidol Trial 4	4	.425	.153	.076
Quinpirole Trial 1	4	.367	.107	.053
Quinpirole Trial 2	4	.374	.053	.028
Quinpirole Trial 3	4	.353	.022	.011
Quinpirole Trial 4	4	.339	.045	.022

Appendix. F. Movement Frequency on 7dpf (Fig. 10)

Treatment and Trial	N	M	SD	SE
Control Trial 1	4	144	150	75
Control Trial 2	4	254	188	93.8
Control Trial 3	4	397	131	75.8
Control Trial 4	4	211	197	114
Haloperidol Trial 1	4	195	123	61.5
Haloperidol Trial 2	4	274	178	89
Haloperidol Trial 3	4	297	102	59
Haloperidol Trial 4	4	112	145	72.5
Quinpirole Trial 1	4	9.25	6.99	3.5
Quinpirole Trial 2	4	20.3	18.4	9.18
Quinpirole Trial 3	4	21.8	17.7	8.87
Quinpirole Trial 4	4	14.3	4.65	2.32

Appendix. G. Total Distance Moved on 9dpf in cm (Fig. 11)

Treatment and Trial	*N*	*M*	*SD*	*SE*
Control Trial 1	*4*	196	69.3	34.7
Control Trial 2	*4*	248	97.6	48.8
Control Trial 3	*4*	300	78.6	39.3
Control Trial 4	*4*	290	106	53
Haloperidol Trial 1	*4*	223	49.7	24.9
Haloperidol Trial 2	*4*	246	80.4	40.2
Haloperidol Trial 3	*4*	322	66.6	33.3
Haloperidol Trial 4	*4*	362	81.4	40.7
Quinpirole Trial 1	*4*	200	77.3	38.6
Quinpirole Trial 2	*4*	250	93.2	46.6
Quinpirole Trial 3	*4*	316	80.1	40.0
Quinpirole Trial 4	*4*	349	112	55.9

Appendix. H. 16-17dpf Distance Results
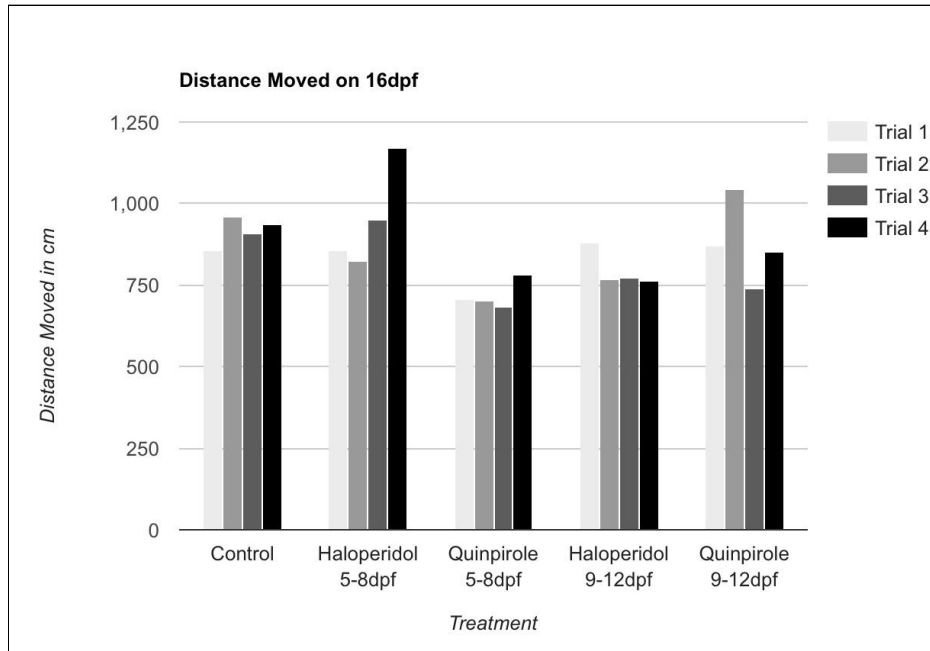
Total Distance Moved on 16dpf across all trials
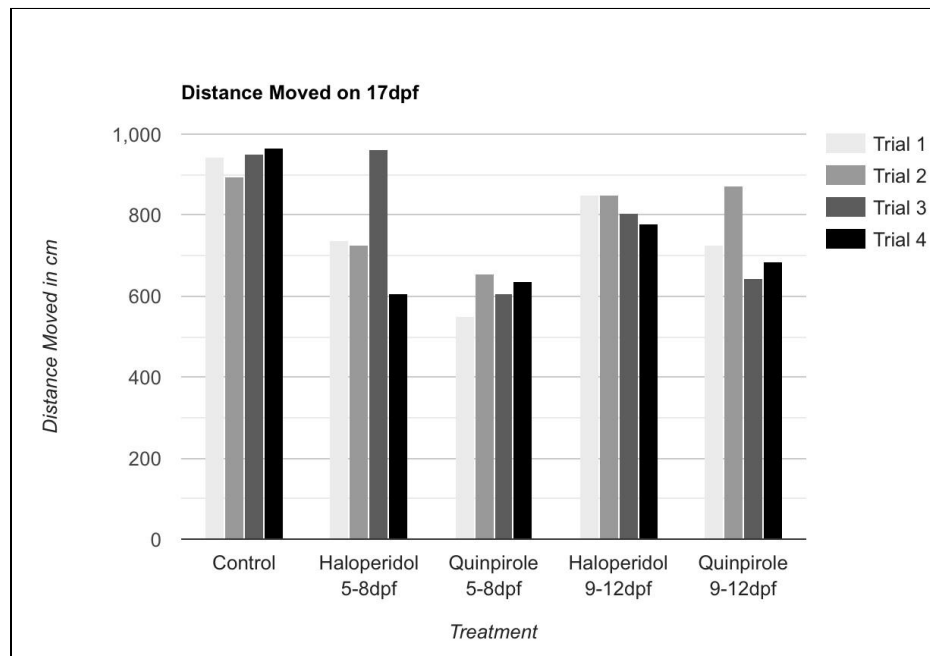
Total Distance Moved on 17dpf across all trials

Appendix. I. 16-17dpf Frequency Results
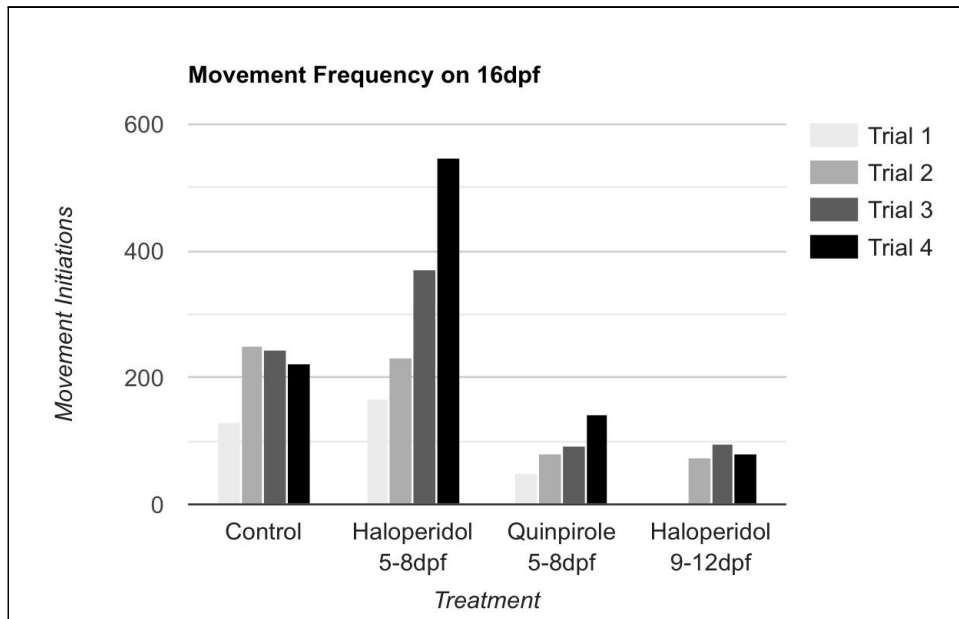
Movement Frequency on 16dpf across all trials
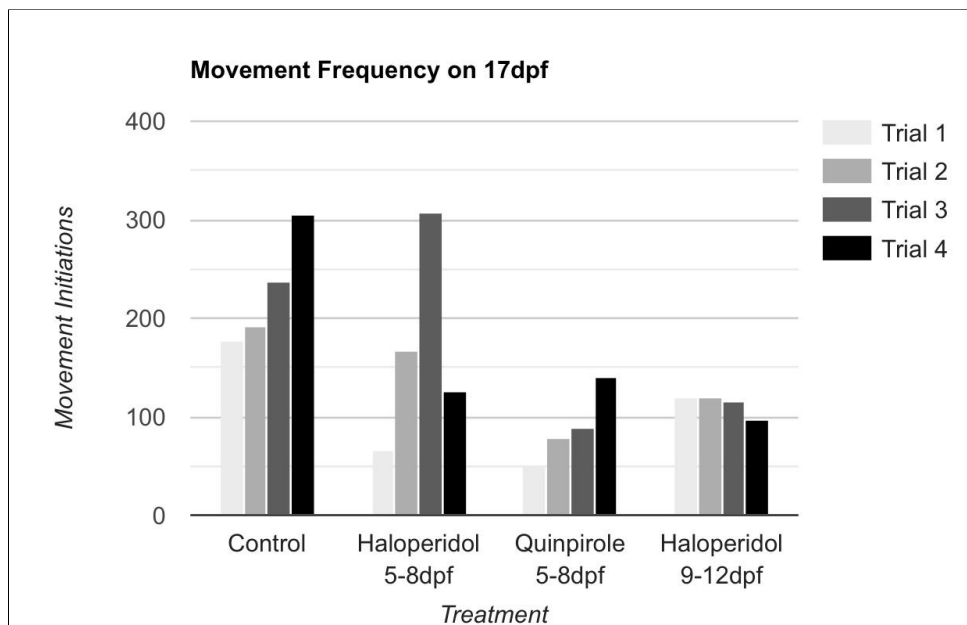
Movement Frequency on 17dpf across all trials

Appendix. J. 16-17dpf Velocity Results
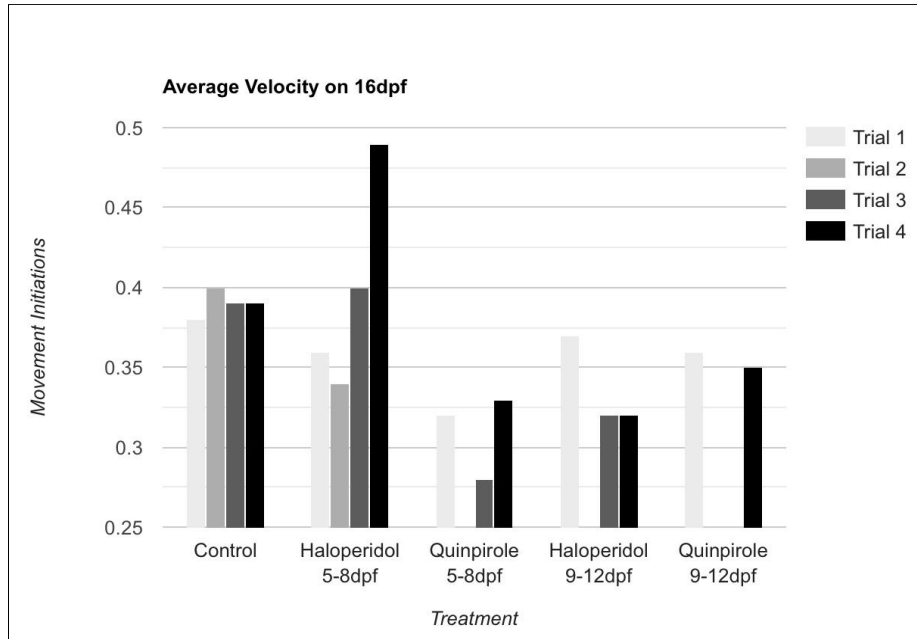
Average Velocity on 16dpf across all trials
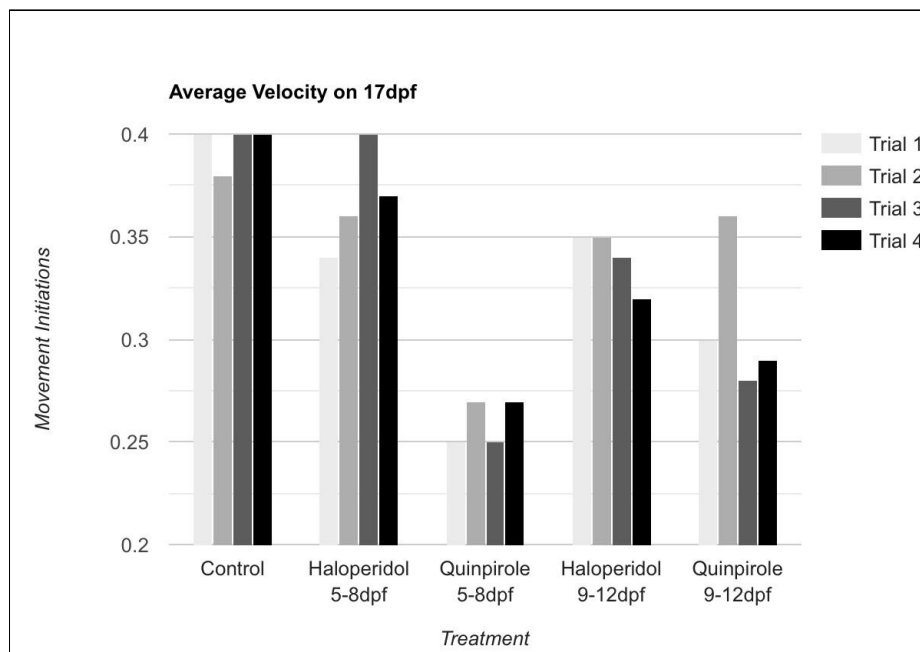
Average Velocity on 17dpf across all trials

Appendix. K. Repeated Measure ANOVA Results 5-8dpf Distance

5 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	166179	3	56393	67.02	< .001
Trial x Condition	20647	6	3441	4.09	0.005
Residual	22718	27	841		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	46242	2	23121	3.05	0.097
Residual	68204	9	7578		

6 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	33886	3	11295	3.033	0.046
Trial x Condition	5705	6	951	0.255	0.953
Residual	100554	27	3724		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	27985	2	13993	2.68	0.122
Residual	47023	9	5225		

7 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	41735	3	13912	4.841	0.008
Trial x Condition	15903	6	2651	0.922	0.495
Residual	77587	27	2874		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	294928	2	14764	3.88	0.061
Residual	342017	9	38002		

8 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	8965	3	2988	1.73	0.184
Trial x Condition	21045	6	3508	2.04	0.095
Residual	46506	27	1722		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	40001	2	20000	1.9	0.205
Residual	94917	9	10546		

Appendix. L. Repeated Measure ANOVA Results 5-8dpf Frequency

5 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	25369	3	8456	5.77	0.003
Trial x Condition	42852	6	7142	4.87	0.002
Residual	39575	27	1466		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	129075	2	64537	5.7	0.025
Residual	101893	9	11321		

6 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	442	3	147	0.232	0.874
Trial x Condition	3057	6	510	0.801	0.578
Residual	17173	27	636		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	2620	2	1310	0.588	0.575
Residual	20045	9	2227		

7 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	73159	3	24386	4.47	0.011
Trial x Condition	49135	6	8189	1.5	0.215
Residual	147262	27	5454		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	392834	2	196417	3.09	0.095
Residual	572393	9	63599		

8 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	12471	3	4157	1.55	0.223
Trial x Condition	19465	6	3244	1.21	0.33
Residual	72190	27	2674		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	37417	2	18708	2.82	0.112
Residual	59638	9	6626		

Appendix. M. Repeated Measure ANOVA Results 5-8dpf Velocity

5 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.052	3	0.017	5.55	0.004
Trial x Condition	0.052	6	0.008	2.79	0.031
Residual	0.084	27	0.003		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.146	2	0.073	2.52	0.135
Residual	0.261	9	0.029		

6 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.054	3	0.018	4.24	0.014
Trial x Condition	0.051	6	0.008	2.03	0.096
Residual	0.114	27	0.004		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.087	2	0.044	3.4	0.08
Residual	0.116	9	0.012		

7 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.116	3	0.386	4.793	0.008
Trial x Condition	0.045	6	0.007	0.928	0.491
Residual	0.217	27			
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.827	2	0.414	3.89	0.061
Residual	0.957	9	0.106		

8 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.019	3	0.006	1.35	0.279
Trial x Condition	0.059	6	0.009	2.09	0.088
Residual	0.127	27	0.004		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.101	2	0.051	1.66	0.243
Residual	0.273	9	0.03		

Appendix. N. Repeated Measure ANOVA Results 9-12dpf Distance

9 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	123868	3	41289	18.422	<.001
Trial x Condition	7250	6	1208	0.539	0.774
Residual	60515	27	2241		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	7373	2	3687	0.17	0.847
Residual	195484	9	21720		

10 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	37145	3	12382	5.15	0.006
Trial x Condition	25569	6	4261	1.77	0.142
Residual	64870	27	2403		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	100713	2	50357	2.5	0.137
Residual	181091	9	20121		

11 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	87003	3	29001	10.59	<.001
Trial x Condition	43931	6	7322	2.67	0.036
Residual	73912	27	2737		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	15120	2	7560	1.51	0.271
Residual	44958	9	4995		

12 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	13710	3	4570	4.01	0.018
Trial x Condition	32421	6	5404	4.74	0.002
Residual	30794	27	1141		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	14281	2	7140	1.29	0.322
Residual	49927	9	5547		

Appendix. O. Repeated Measure ANOVA Results 9-12dpf Frequency

9 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	203446	3	67815	14.55	0.965
Trial x Condition	6298	6	1050	0.225	
Residual	125842	27	4661		
	Between Subjects Effects				
	SS	df		F	p
Condition	26633	2	13316	0.265	0.773
Residual	453100	9	50344		

10 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	129914	3	43305	6.48	0.002
Trial x Condition	67301	6	11217	1.68	0.165
Residual	180359	27	6880		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	157534	2	78767	1.41	0.294
Residual	504466	9	56052		

11 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	44854	3	14951	12.314	<.001
Trial x Condition	5977	6	996	0.82	0.564
Residual	32781	27	1214		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	1106	2	553	0.098	0.907
Residual	50339	9	5593		

12 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	4041	3	1347	5.88	0.003
Trial x Condition	3948	6	658	2.87	0.027
Residual	6189	27	229		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	1961	2	980	0.874	0.45
Residual	10097	9	1122		

Appendix. P. Repeated Measure ANOVA Results 9-12dpf Velocity

9 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.324	3	0.108	18.173	<.001
Trial x Condition	0.023	6	0.004	0.65	0.69
Residual	0.16	27	0.006		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.016	2	0.008	0.133	0.877
Residual	0.534	9	0.59		

10 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.104	3	0.034	5.19	0.006
Trial x Condition	0.071	6	0.019	1.78	0.141
Residual	0.18	27	0.068		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.28	2	0.14	2.51	0.136
Residual	0.503	9	0.055		

11 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.251	3	0.083	15.624	<.001
Trial x Condition	0.03	6	0.005	0.936	0.486
Residual	0.144	27	0.005		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.026	2	0.013	0.649	0.546
Residual	0.182	9	0.02		

12 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.044	3	0.014	5.91	0.003
Trial x Condition	0.074	6	0.012	4.91	0.002
Residual	0.068	27	0.002		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.03	2	0.015	1.05	0.388
Residual	0.127	9	0.014		

Appendix. Q. Repeated Measure ANOVA Results 16-17dpf Distance

16 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	6080	3	2027	0.703	0.554
Trial x Condition	31156	12	2596	0.901	0.552
Residual	164341	57	2883		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	38962	4	9741	0.731	0.582
Residual	253053	19	13319		

17dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	1230	3	410	0.19	0.903
Trial x Condition	20538	12	1711	0.795	0.654
Residual	122776	57	2154		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	78730	4	196882	1.14	0.367
Residual	327307	19	17227		

Appendix. R. Repeated Measure ANOVA Results 16-17dpf Frequency

16 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	8528	3	2843	1.81	0.155
Trial x Condition	27082	12	2257	1.44	0.175
Residual	8290	57	1566		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	44116	4	11029	1.79	0.174
Residual	117389	19	6178		

17 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	0.005	3	0.0017	0.397	0.756
Trial x Condition	0.028	12	0.0024	0.532	0.885
Residual	0.256	57	0.0045		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	0.22	4	0.055	1.23	0.33
Residual	0.846	19	0.044		

Appendix. S. Repeated Measure ANOVA Results 16-17dpf Velocity

16 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	6080	3	2027	0.703	0.554
Trial x Condition	31156	12	2596	0.901	0.552
Residual	164341	57	2883		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	38962	4	9741	0.731	0.582
Residual	253053	19	13319		

17 Dpf	Within Subjects Effects				
	SS	df	Mean Square	F	p
Trial	3611	3	1204	1.86	0.146
Trial x Condition	7803	12	650	1.01	0.456
Residual	36842	57	646		
	Between Subjects Effects				
	SS	df	Mean Square	F	p
Condition	22481	4	5620	1.7	0.192
Residual	62830	19	3307		

Appendix. T. Pilot Study 1

This study was the first pilot study of this research aiming to find preliminary evidence of

D2 modulation having an effect on activity. Embryos were cared for in until 5dpf wherein they

were transported to an incubator in the Behavioral Neuroscience lab in Preston Hall. The larvae

were immediately separated into control and treated groups, with eight and six subjects

respectively. After an hour of acclimation, the six treated condition larvae were placed into a

16µ/mol solution of haloperidol for 15 minutes (from approximately 11:35-11:50a.m.).

Following treatment, these six larvae were placed along with six control subjects into a 4x3

well-plate of individual arenas filled with untreated EW water. The arenas were placed inside of

the DanioVision observation chamber and their movement was tracked and analyzed using the

Noldus Ethovision XT software. This dosing and tracking procedure was repeated at the same

time for dpf 6, 7, and 8. On days 18 and 19 post fertilization the subjects were tracked once again

without any drug treatment to assess if differences in movement behavior were observable 10

days after the final drug administration. The results displayed conflicting results as on day 18dpf

the treated group (*M=1402.13, SD=267.5)* had in comparison to the control (*M=982.92,*

SD=187.5) a significantly higher level of movement 10 days after final drug administration *t*(8)

= 2.57, *p* 0.033. However, the following day at 19dpf the treated group (*M=1721.64, SD= 188.2)*

did not display the same effect *t*(8) = .22, *p* = .82 as the control group (*M=1685.64, SD=260.3)*

did not differ significantly from the treated group's movement distance.

Appendix. U. Pilot Study 2

This study was designed to determine if observable differences in behavior occurred after drug exposure and assess proper duration of exposure. This was essentially to assess if the proposed procedure was viable and to practice it. The subjects of this pilot study did not undergo early stage modulation rather all subjects were in a controlled environment and uniform environment until 21dpf where upon six of the larvae were placed into a 16μ/mol solution of haloperidol, three for 15 minutes and three for 30 minutes. This solution was created using a stock of 12mg Haldol with 8ml of DMSO in 40 mL of EW and then diluted by removing 4mL of stock and adding 196mL of EW to create the desired 16μ/mol solution which. The dosage was modeled after the procedure utilized by (Irons et al,, 2013). The movement distance of the treated larvae in the 30 min group (*M=1177.51, SD=163.31*) in comparison to control levels (*M=631.32, SD=244.6*)were found to be significantly greater, $t(22) = 6.16, p = < 0.001$. The 15 minute group (*M=858.27, SD=226.81*) also displayed an increased level of movement $t(16) = 3.24, p = 0.005$) from controls (*M=537.02, SD=165*). The greater significance of the 30 min dosage led to the decision to use this dosage in the final experiment to more reliably link behavioral differences to D2 modulation.

Appendix. V. Pilot Study 3

The third pilot study was conducted to test the experimental design and determine if there were any flaws with the procedure. The embryos were bred inside and remained there until 4dpf wherein they were transferred and separated into six groups of 15 subjects (4 experimental groups and 2 control groups). The following day subjects from both 5dpf-8dpf groups were dosed for a half an hour with 16μ/mol of haloperidol and quinpirole respectively. During this procedure the haloperidol group was dosed from approximately 11:30pm to 12:00pm and were tested for locomotor behavior in the DanioVision lightbox with six treated and 6 control subjects. The quinpirole 5dpf- 8dpf group was also then dosed from approximately 12:00pm to 12:30pm and tested afterwards, again with 6 treated and 6 control. This procedure was repeated from days 5 through 8 after which the 9dpf-12dpf groups underwent an analogous procedure for the next 4 days. Following the final drug administration the larvae were maintained and cared for for the next 11 days. Unfortunately, several experimental groups had a significant die off near the end of this period and thus, the data gathered post drug administrations is sparse. While this study was unsuccessful it served to polish the final study's experimental design.