THE EFFECTS OF SOCIAL STATUS ON DOPAMINERGIC REGULATION OF NEURAL CIRCUIT ACTIVATION AND BEHAVIOR

By

Katie N. Clements

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Director of Thesis: Dr. Fadi A. Issa

Major Department: Biology

Social hierarchies can be observed within communities across many species and allow for proper allocation of resources. When forming social hierarchies, animals that display the most aggressive behaviors generally emerge as dominant, while less aggressive animals are relegated to a subordinate role. The aim of this study is to address the neural bases of social regulation using zebrafish (*Danio rerio)* as a model organism. When paired, zebrafish form dominance hierarchies that consist of socially dominant and subordinate fish. To better understand the effects of social dominance on nervous system function we investigated the influence of social experience on the escape and swim behaviors. Using a non-invasive technique of recording field potentials, we monitored escape and swimming behavior between fish of known social status. We showed that social status affects neural activation underlying swimming and escape behaviors. Subordinates favor escape over swim, while dominants favor swim over escape. We hypothesized that a neuromodulator associated with social regulation and aggression, dopamine (DA), may influence the activation of the two underlying neural circuits responsible for these behaviors in a social status-dependent manner. To test this hypothesis, we initially looked at whether the supply of DA influenced differences in swimming and escape behavior. We augmented levels of DA through injection and observed no significant changes in the escape or swimming behavior of dominants or subordinates. Next, we determined if the interpretation of

DA, via DA receptors, influenced the status-dependent behavioral differences. We manipulated the activation of DA receptors through injection of DA specific agonists and antagonists. First, antagonizing the dopamine 1 receptor (D1) decreased dominant swimming frequency and increased escape probability, while having no effect on either behavior in subordinates. Activating the D1 receptor caused no changes in escape probability or swimming frequency in either social phenotype. Second, neither application of dopamine 2 receptor (D2) agonist nor antagonist significantly altered escape probability in either social phenotype; however, blocking the D2 receptor reduced dominant swimming frequency. Finally, antagonizing the dopamine 3 receptor (D3) lowered subordinates' probability of escape with no change in swimming frequency, while showing no effect on dominant behavior. Activating the D3 receptor had no effect on dominant or subordinate escape behavior, but decreased dominant swimming. Taken together, these results suggest that the social status-dependent differences in escape and swimming behaviors of zebrafish may be influenced by dopamine receptor activation.

THE EFFECTS OF SOCIAL STATUS ON DOPAMINERGIC REGULATION OF NEURAL CIRCUIT ACTIVATION AND BEHAVIOR

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Katie N. Clements

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By

Katie N. Clements

APPROVED BY:

DIRECTOR OF
THESIS: _____
Fadi A. Issa, PhD

COMMITTEE MEMBER: _____
Stefan Clemens, PhD

COMMITTEE MEMBER: _____
Elizabeth T. Ables, PhD

CHAIR OF THE DEPARTMENT
OF BIOLOGY _____
Jeffrey S. McKinnon, PhD

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

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LIST OF ABBREVIATIONS

5-HT	Serotonin
Co-IP	Co-immunoprecipitation
COMT	Catechol-O-methyltransferase
CPG	Central pattern generator
D1	Dopamine 1 Receptor
D2	Dopamine 2 Receptor
D3	Dopamine 3 receptor
DA	Dopamine
DAT	Dopamine active transporter
GPCR	G-protein coupled receptor
i-IN	inhibitory interneuron
M-Cell	Mauthner Command Neuron
MN	Motor neuron
PKA	Protein kinase A
VIIIth Nerve	Vestibulocochlear sensory nerve

GENERAL INTRODUCTION

Animal behavior in a social setting is dependent on the integration of internal physiological processes with the salience of external stimuli (O'Connell & Hoffmann, 2012). Perturbation of either of these mechanisms leads to a modification in behavioral output. One factor that may influence these mechanisms is the rank of the animals within their community. Social hierarchies permeate the animal kingdom and are established through aggressive interactions. The most aggressive animal often emerges as dominant, while others in the community occupy lower social ranks and are considered subordinates. Relationships within a hierarchy can influence how each animal behaves in a social context. It is important to understand the relationship between social rank and underlying neural function to elucidate how these changes can influence behavioral output.

Social hierarchies have been observed in invertebrate and vertebrate species, and how these social relationships influence behavior and physiology of animals have been extensively studied (Johnsson, 1997; Issa et al., 1999; Sapolsky, 2004; Paull et al., 2010; Oliveira et al., 2011; O'Connel & Hofmann, 2012; So et al., 2015; Platt et al., 2016). Previous studies have focused on the relationship of social status and different aspects of neural function, including neuronal apoptosis (Sapolsky, 2004), neuronal morphology (Joëls et al., 2007), and cognition (So et al., 2015). Another component of neural function is neuromodulation. There are two neuromodulators, DA and serotonin (5-HT), that are associated with aggression, mood, movement, and social regulation. The effects of 5-HT on social behavior and hierarchy formation have been well characterized (Larson & Summers, 2001; Cubitt et al., 2008; Chiao, 2010; Kiser et al., 2012) and 5-HT input on neuronal function (Korn & Faber, 2005; McLean & Fetcho 2004). In contrast, the specific role of DA neuromodulation on hierarchy formation and

changes in neuronal signaling underlying behavior remains an avenue to be explored. Dopaminergic nuclei project to striatal and limbic system structures (Fiorentini et al., 2008) affecting movement and emotional and cognitive processes. Previous research has shown dopaminergic involvement with aggression (Filby et al., 2010), motivation (Chiara, 1995; Depue & Collins, 1999; Hamid et al., 2016), and hierarchy formation (Watanabe & Yamamoto, 2015). As a result, we chose to focus on how social status might modulate dopaminergic input affecting neural circuits and behavior.

Zebrafish as a Model Animal to Investigate the Neural Bases of Hierarchy Formation

Zebrafish are becoming more widely accepted as a model for studying adult nervous system function. Although best known for their advantages in developmental biology (Peng et al., 2012), they show conserved brain structures and neural mechanisms when compared to higher vertebrates (Yamamoto & Vernier, 2011). This makes them an attractive model for studying fundamental neuronal processes.

Zebrafish demonstrate aggressive behaviors (Paull et al., 2010; Oliveira et al., 2011). Competition for resources, such as food, shelter, and mating preference forces animals to engage in physical aggression to establish a hierarchy. This ultimately allows for stability of the community (Larson et al., 2006; Oliveira et al., 2011; Pavlidis et al., 2011). Typical displays of aggression between fish are chasing and biting. The animal that displays the most aggressive behaviors emerges as a dominant. In contrast, fish that are the target of the aggressor display a submissive behavior, retreating, and are classified as subordinate. We are able to exploit these behaviors in a controlled environment to study how a stable social status affects neural function and behavior.

When studying neural mechanisms underlying behavior, zebrafish provide distinct advantages in that they produce a well-characterized escape response mediated by the activation of a pair of command neurons, named Mauthner neurons (M-cells). M-cells have been extensively studied and much is known of their physiology (Kashin et al., 1974; Eaton et al., 1977; Zottoli, 1977; Zottoli et al., 1987; O'Malley et al., 1996; Ali et al., 2000; Venables & Ripley, 2002; Severi et al., 2014). M-cell axons innervate contralateral motor neurons (MNs). An activated M-cell will elicit the firing of contralateral MNs, which will produce a bend away from the perceived threat. This behavior is easily identified and quantified using non-invasive electrophysiological recordings of muscle potentials.

In addition to escape, zebrafish produce a more phasic behavior, swimming. The swimming circuit involves the mesencephalic locomotor region (MLR), which projects to spinal central pattern generators (CPGs). CPGs activate slow motor neurons (Slow MNs), allowing the fish to switch back and forth from left and right motor neuron activation. This behavior is distinguishable from the M-cell escape response in that the muscle potentials and physiological recordings are more variable in size and duration. Swimming and escape circuit activation happen independently (Miller et al., 2017). This allows us to determine how social status affects decision making between two competing circuits.

The neuromodulators DA and serotonin (5-HT) innervate the proximity of the Mauthner neurons and also modulate the swimming circuit (Oda et al., 1998; McLean & Fetcho, 2004; Larson et al., 2006). Research has primarily focused on social status modulation of 5-HT input on the M-cells and the escape response (Yeh et al., 1996; Korn & Faber, 2005; Neumeister et al., 2010; Whitaker et al., 2011), and little is known of DAs involvement in social status-dependent regulation of the M-cells. DAs role in the swimming circuit has been studied, and we know that

levels of DA can influence locomotor activity (Li et al., 2010; Lambert et al., 2012; Gupta et al., 2014). However, whether social status influences DAs involvement in locomotor activity underlying swimming behavior has not been determined. Here, we investigated how social status affects DA regulation of neural circuits underlying both swimming and escape behavior in zebrafish. Our hypothesis was that changes in DA pathway signaling played a role in modifying swimming and escape behaviors of zebrafish as a result of social hierarchy formation.

Thesis Outline

The primary objective of this study was to understand how social status modified swimming and escape circuits in zebrafish by looking at changes in dopaminergic pathway signaling. In chapter one, we found that social hierarchies influenced underlying neural activity that shape the behavior of the Mauthner escape and swim circuits in dominant and subordinate animals. More specifically, subordinate animals increased showed activation of escape over swim circuits, while dominants showed higher activation of swim over escape circuits. These results confirmed that social experience affects the activation dynamics of these two circuits underlying behavioral output.

In chapter two we determined the role of DA in modulating the status-dependent changes in escape and swim circuits. Miller et al. (2016) showed that gene expression of DA receptors are differentially regulated by social rank. To test whether status-dependent differences in DA receptor expression influenced differences in escape and swim behaviors, we pharmacologically manipulated DA signaling through systemic injection of receptor agonists and antagonists. We presented evidence that activation of specific DA receptors could modify the social status-dependent activation of the escape and swim behaviors. With these results, we gain a better understanding of how DA signaling may contribute to differences in behavioral output of

dominant and subordinate fish.

The discussion consolidated the results of chapters one and two on how social status affected the balance of activation dynamics of the escape and swim circuits through changes in dopaminergic signaling. We proposed a model to explain how DA may regulate escape behavior in dominant and subordinate animals. With our results, we found that there were still gaps in our understanding of how DA signaling may regulate the escape and swim behaviors. In turn, we proposed future experiments to help bridge these gaps and create a better understanding of how DA receptors influence the escape and swim circuits underlying status-dependent changes in behavior.

GENERAL METHODS

Animal maintenance. Experiments followed the National Institutes of Health guidelines and East Carolina University's Institutional Animal Care and Use Committee approval (AUP #D320). Adult wild type AB zebrafish (*Danio rerio*) were maintained at 28°C, pH 7.3 under a 14h/10h light/dark cycle and fed three times daily.

Dominance hierarchy formation and behavioral observations. Male fish were removed from communal housing tank and were isolated in individual tanks (23 x 13 x 6cm) for one week. After one week of isolation, males were paired (size and age-matched) in a novel housing tank. Social interactions between paired males were observed daily for the two week pairing period for 5 minutes. Displays of aggression (attacks: bites and chase) and submission (retreats) were recorded on paper.

Measuring swimming activity and place preference during pairing. Pairs were filmed daily (early afternoons) for 1 minute to monitor changes in their swimming activity using a Canon Camcorder (Digital video ZR500). Videos were digitized and movement (distance traveled over one minute periods) of each fish was analyzed using NIH ImageJ software Manual Tracking plugin. Instances when animals were interacting with one another resulted in the exclusion of those video frames from analysis. Total tracked distance was normalized to the number of remaining video frames. For place preference, video recordings of animal movement were tracked using the Manual Tracking plugin for ImageJ to extract XY coordinates within the housing tank. Videos were down-sampled to 3 frames/sec and coordinates for dominants, and subordinate animals were loaded into R software using a custom script. XY coordinates encompassing periods of social interactions were removed from the analysis. Filled contour

plots combining data of all dominant or subordinate animals were produced using 2D kernel density estimations generated by the kde2d function of the MASS package (CRAN repository) (Venables & Ripley, 2002). The algorithm disperses the mass of the empirical distribution function over a regular grid of 512 points and uses the fast Fourier transform to convolve this approximation with a discretized version of the kernel followed by linear approximation to evaluate the density at the specified points. Density data was converted into a heat-map probability plot to facilitate illustration of the data set for both social phenotypes.

Measurement and analysis of swimming and escape field potentials. Animals were placed in the testing chamber (Figure 2A) and allowed to acclimate for 30 minutes. For chapter one, field potentials of burst swimming activity were recorded continuously for 1 minute. Swim bursts were detected and sorted using the "threshold" search tool of the Clampfit software (Molecular Devices). Detected bursts were processed and verified according to the following criteria: a burst was included if it was larger than 12 mV in total amplitude and 50-200 ms in duration (Figure 2B). Markers were assigned to each burst at half-width of the burst (usually at the peak burst amplitude) for all bursts during the 60 second recording period. For chapter two, field potentials of burst swimming activity were detected for a ~10 second recording period. Burst were processed as stated above. M-cell detection was processed by the latency of the response from the stimulus (<15ms) and presence of a large phasic field potential (Figure 2B). Data was tabulated into Microsoft Excel and analyzed using Prism (GraphPad software Inc., San Diego, USA). All values are provided as mean and ± SEM unless otherwise stated.

Pharmacology and drug administration. The following drugs were used for manipulation of the dopaminergic signaling pathway: L-DOPA; SCH23390 (D1 antagonist), Dihydrexidine (D1

agonist); Raclopride (D2 antagonist), Bromocriptine (D2/D3 agonist); SB 277011A (D3 antagonist), Pramiprexole (D3 agonist). All agonists and antagonists were purchased from Tocris Bioscience. Capillary tubing (1.0mm OD x 0.5 mmID, 100mm Each – 250/PKG, 25 mm total length) were pulled using Flaming/Brown Micropipette Puller – Model P-87 from Sutter Instrument Co. A stock solution of 5mg/ml of each drug was prepared and loaded into capillary injection needle. The tip of needle was broken with razor and needle was placed in Pneumatic PicoPump PV 820 for administration. Dominant and subordinate fish were anesthetized in a 0.18mg/ml solution of Tricaine-S. After anesthesia, each fish was placed under a microscope on KimTech Kimwipe and injected caudally, roughly 1 cm from tailfin, with 5 puffs (2 µl total volume) of the respective drug. Fish were placed back in pairing tank with a divider separating the tank to allow fish to recover undisturbed. After 90 minutes of recovery, fish were relocated to behavioral testing chamber with an additional 30 minutes for recovery and acclimation.

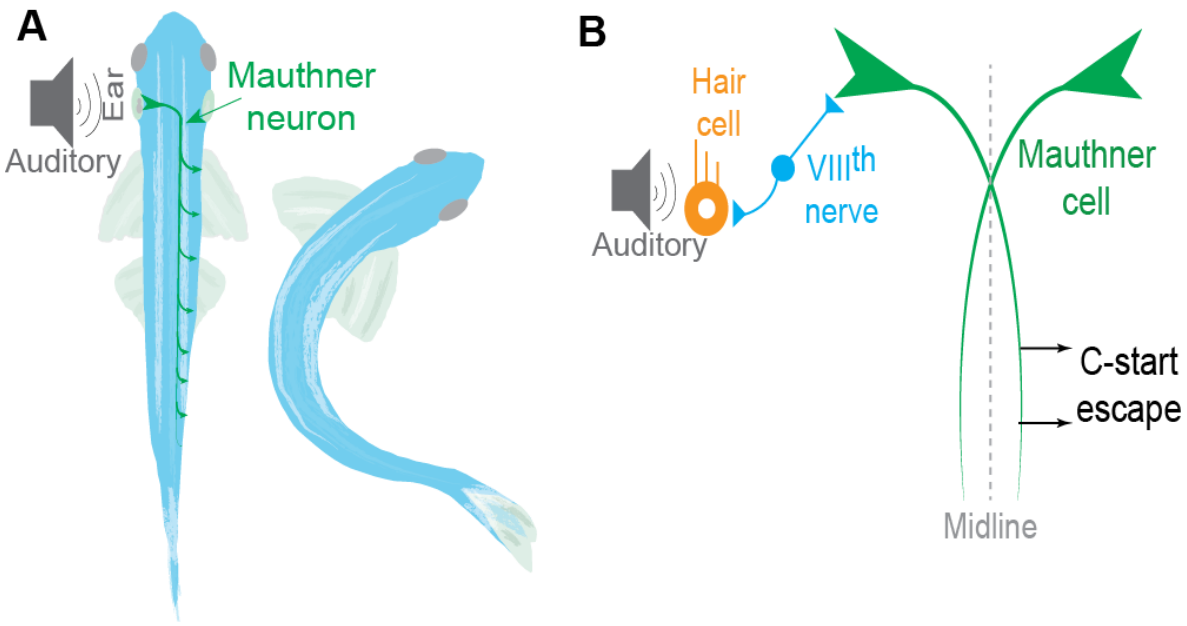

Figure 1 - Schematic illustrations of the M-cell escape circuit
A) Zebrafish startle response is activated by auditory stimuli. This behavior is mediated by the Mauthner neural circuit. Activation of the M-cell is necessary for escape. M-cell innervates spinal cord motor neurons. B) Schematic illustration of the M-cell escape circuit. M-cell escape response receives input from the VIIIth sensory nerve.

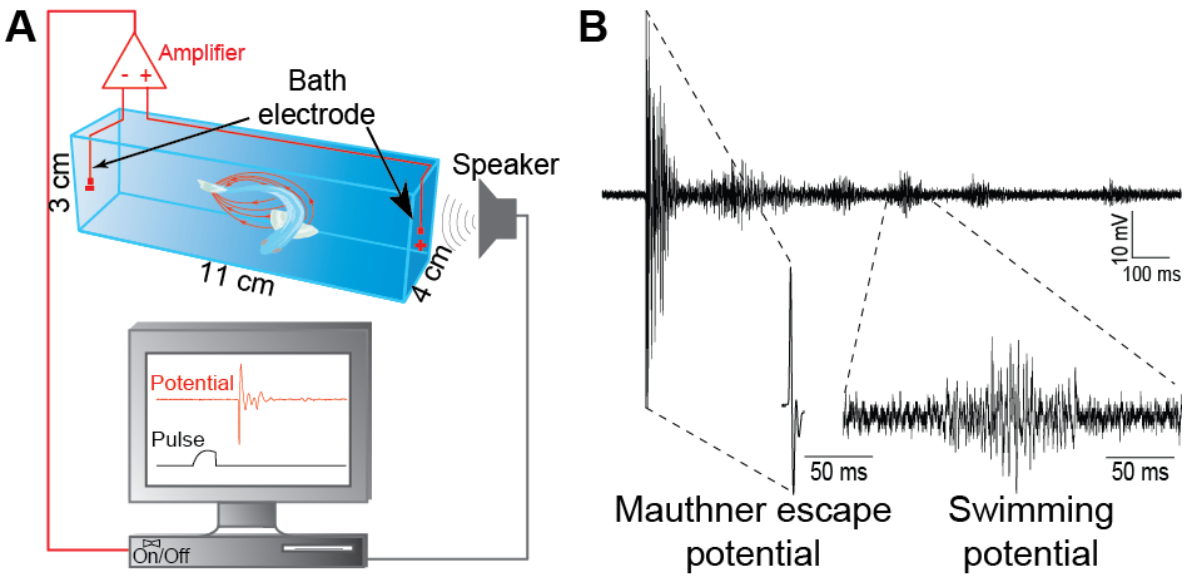

Figure 2 – Behavioral quantification by non-invasive electrophysiology
A) Schematic of testing and recording chamber used to measure C-start escapes and swimming activity. A pair of bath electrodes is placed on either side of the testing chamber. Bath electrodes detect neuromuscular field potentials generated as the M-cell escape response is activated. M-cell escape is activated by an auditory pulse. Field potentials and stimuli are time-locked and digitally recorded. B) A representative example of a phasic field potential recording recorded during activation of the C-start escape response mediated by the M-cell. The Mauthner escape potential is often followed by an immediate swimming potential that are significantly lower in amplitude.

CHAPTER I

SOCIAL STATUS-MEDIATED CHANGE IN TWO NEURAL CIRCUITS

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*Equal first authorship.

Introduction

Choosing proper behavior can be critical to the survival of an animal. It is important to understand how social rank may modify an animal's behavioral output. In this study, we investigated how social status influences decision-making between two well-characterized behaviors, the Mauthner mediated escape response and swimming. These two behaviors have distinct underlying neural circuits that do not occur simultaneously (Miller et al. 2017). In addition, the morphological and functional aspects of these behaviors have been extensively studied (Ali, Drapeau, & Legendre, 2000; Eaton et al., 1977; Kashin et al., 1974; O'Malley et al., 1996; Venables et al., 2002; Zottoli, 1977; Zottoli et al., 1987).

The neural basis of Mauthner startle escape behavior has previously been investigated in teleost fish (Eaton et al., 2001; Korn & Faber, 2005). This response is controlled by a distinct reticulospinal neural network centered around the M-cells. These neurons receive unilateral sensory input and project their axons contralaterally across the midline to innervate spinal cord motor neurons. One input-type is auditory from the vestibulocochlear nerve (VIIIth nerve)

(Kohashi & Oda, 2008). This synapse is a mixed synapse consisting of both electrical, through gap junctions, as well as chemical, through excitatory glutamatergic neurotransmission (Medan & Preuss, 2014; Pereda et al., 2005; Pereda et al., 1994; Yao et al., 2014). When activated, this circuit elicits a reflexive contraction of the muscles, resulting in a bend away from the perceived threat (Figure 1) (Canfield, 2003; Eaton & Emberley, 1991; Preuss et al., 2006; Zottoli, 1977).

The swimming circuit is more distributed yet still organized hierarchically (Severi, et al., 2014; Thiele et al., 2014; Wang & McLean, 2014). Stimulation of the mesencephalic locomotor region (MLR) evokes swimming (Canfield 2003; Kashin et al., 1974). Descending outputs from the MLR are transmitted to reticulospinal neurons located in the mid- and hindbrain regions. Their axonal projections excite spinal CPGs that drive coordinated rhythmic swimming activity (Deliagina et al., 2002).

Using non-invasive recording, we monitored behavioral patterns in freely behaving animals (Issa et al., 1999). We show that these behaviors and the activation of their underlying neural circuits are susceptible to status-dependent modulation and we present a simple schematic to illustrate our findings.

Results

Zebrafish form stable social hierarchies. Paired zebrafish quickly form stable dominance relationships. We observed the social agonistic interactions of paired adult male zebrafish daily and counted aggressive (attacks) and submissive (retreats) behaviors performed by each fish for the pairing period. Dominance relationships were established by the third day of interactions and remained stable for the remainder of two weeks of observation (Figure 3). During this period, the dominant animal for each pair performed most of the aggressive chasing and biting behavior (Figure 3A, top). In contrast, subordinates performed more submissive retreating behaviors (Figure 3A, bottom). These results suggest that zebrafish form stable social hierarchies as a consequence of aggressive interactions between animals during the two week pairing period.

Social status-dependent regulation of swimming behavior. As animals formed their dominance hierarchy, swimming patterns changed within the housing tank. Figure 3B shows the spatial probability of dominants and subordinates within the tank. Dominant animals swam freely within the tank, while subordinates maintained their position in the bottom corner. To quantify this observation, we measured the average swimming distances for each day of pairing for 1-minute over 14 consecutive days (Figure 3C). We performed a two-way ANOVA to test differences in swimming activity by social rank (factors: group and days). There were significant main effects of group [$F(2, 535)=2.11e+2$, $p<1.0e-16$, Figure 3C] and days [$F(14, 535)=3.12$, $p=1.02e-4$, Figure 3C]. There was also an effect of interaction between group and days [$F(28, 535)=2.93$, $p=1.21e-6$, Figure 3C]. In particular, we observed that the normalized swim distance of dominants was significantly longer than those of group-housed and

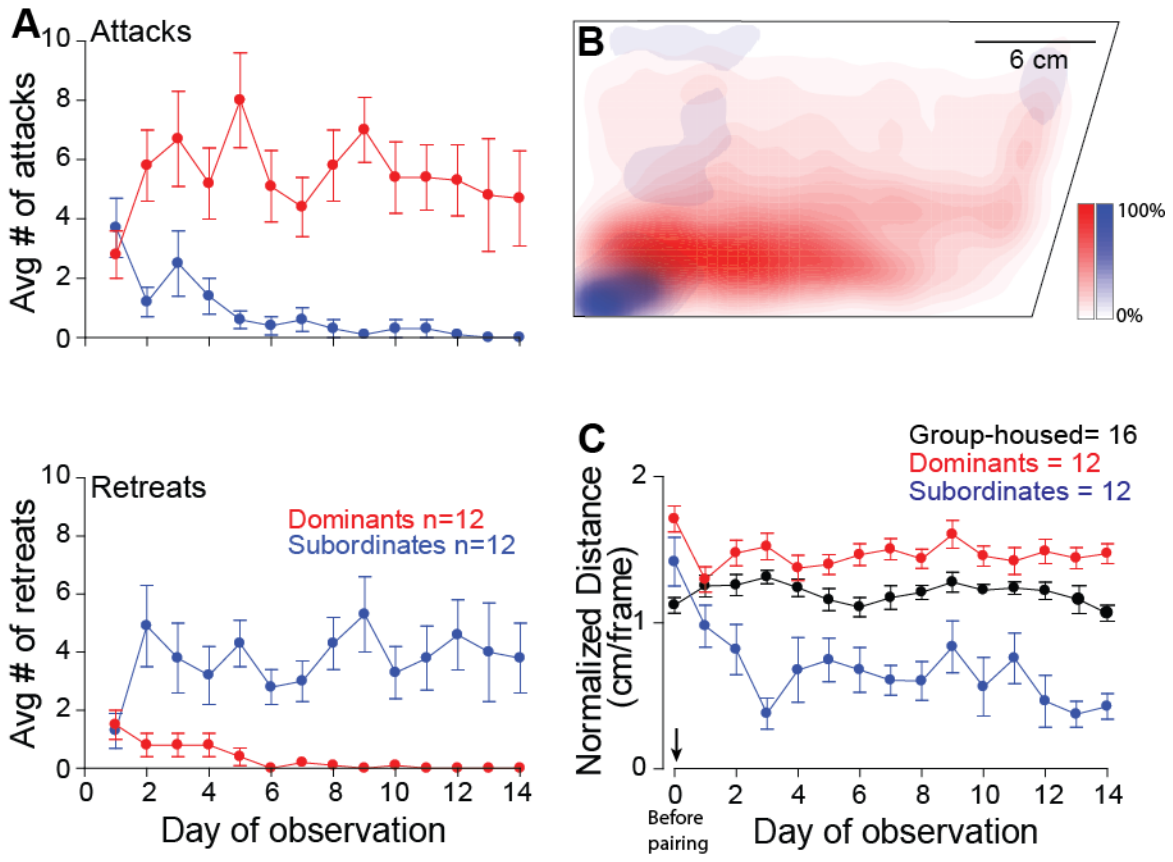

Figure 3 – Zebrafish form stable social hierarchies with divergent behavior

Adult male zebrafish form stable social relationships and behavior patterns diverge significantly as social relationships solidify. A) Social interactions are characterized by aggressive behaviors (attacks, top graph) performed predominantly by dominants and submissive behaviors (retreats, bottom graph) displayed mainly by subordinates. B) Social status affects swimming activity. Kernel heat-map estimation plot of swimming activity over a 1-minute period of filming for Dominants (red) and subordinates (blue) on day 12 post pairing (n=12 Dominants and 12 subordinates). C) Quantification of filmed swimming activity [distance traveled (cm)/frame] for all animals tested during a 1-minute period, each day for a 14-day pairing period. Video frames during which the animals were engaged in aggressive interactions were excluded from analysis. Total tracked distance was normalized by the number of remaining video frames. Day 0 marks observations of animals before they were paired (Before pairing: arrow).

subordinate animals [Tukey HSD, p≤9.56e-10, Figure 3C] and the normalized swim distance of group-housed animals was also significantly longer than that of subordinates [Tukey HSD, p=9.56e-10, Figure 3C]. These results suggest that as a dominance hierarchy is formed, swimming behavior of both social phenotypes changes where subordinates show a decline in swimming behavior compared to controls, while dominants show an increased swimming activity (Figure 3C, B; n=12 for dominants and subordinates each, and n=16 for group-housed animals).

Secondly, we measured the animals' swim burst activity. Animals were tested individually by placing them in the testing chamber described above and recorded spontaneous swimming bursts field potentials for 1 minute after a 30 minute period of acclimatization on day 12 of pairing (Figure 4A). We observed a divergence in swimming behavior between the two social phenotypes. Dominant animals increased swim burst activity more than group-housed and subordinate animals (Figure 4B, C). We performed a two-way ANOVA (factors: group and time bin) to compare the average number of bursting swim activities. There was significant main effect of group [$F(2, 480)=5.45e+1$, $p<1.0e-16$, Figure 4C] while there was no effect of time bin [$F(11, 480)=9.70e-1$, $p>0.05$, Figure 4C]. We observed that the average number of bursting swim of dominants was significantly higher than those of group-housed and subordinates [Tukey HSD, p≤1.36e-9, Figure 4C] and the average number of bursting swim of group-housed was significantly higher than that of subordinates [Tukey HSD, p=1.19e-4, Figure 4C]. We also compared the total number of swim bursts with one-way ANOVA (factor: group). We found a significant main effect of group [$F(2, 40)=1.49e+1$, $p=1.48e-5$, Figure 4C]. Dominants showed a significantly higher number of bursting swim compared to group-housed and subordinate animals [Tukey HSD, p≤4.87e-3, Figure 4C, D]. These results suggest that social interactions

have impacted the swim circuit of both dominant and subordinate animals in different ways. Social dominance caused an increase while social submission led to a decrease in the activity of the swim circuit.

Social status affects the escape response. To determine whether social experience affects the activation threshold of the M-cell escape, we tested the animals' sensitivity to auditory pulses of randomized decibel intensities. At low decibels, both social phenotypes displayed similarly low response probabilities (Figure 5A). However, at higher decibels, subordinates were significantly more sensitive and were more likely to respond to auditory pulses compared to dominants and group-housed animals (Figure 5B). Subordinate animals' response probability reached the V50 at 82.73 dB compared to dominants of 86.86 dB and group-housed animals 85.30 dB (One-way ANOVA with Newman-Keuls Multiple Comparison Test, two-tailed, *P=0.0091* at 85 dB and *P=0.0344* at 90 dB). Data was curved fitted with a non-linear regression with Boltzmann Sigmoidal curve fit; Goodness of fit R^2 for group-housed= 0.9868, Dominants= 0.9924, Subordinates= 0.9926; group-housed n=20; Dominants n=23; Subordinates n=23). At supra-threshold stimuli (95dB and higher) the response probability of animals plateaued to similar levels. Comparison of the sensitivity curves of dominant and subordinate animals to group-housed shows that paired-wise interactions had a significantly bigger impact on the response sensitivity of socially subordinate animals compared to dominants (Figure 5B). Our results suggest that the activation threshold of the M-cell in subordinates decreased significantly compared to both dominant and group-housed animals.

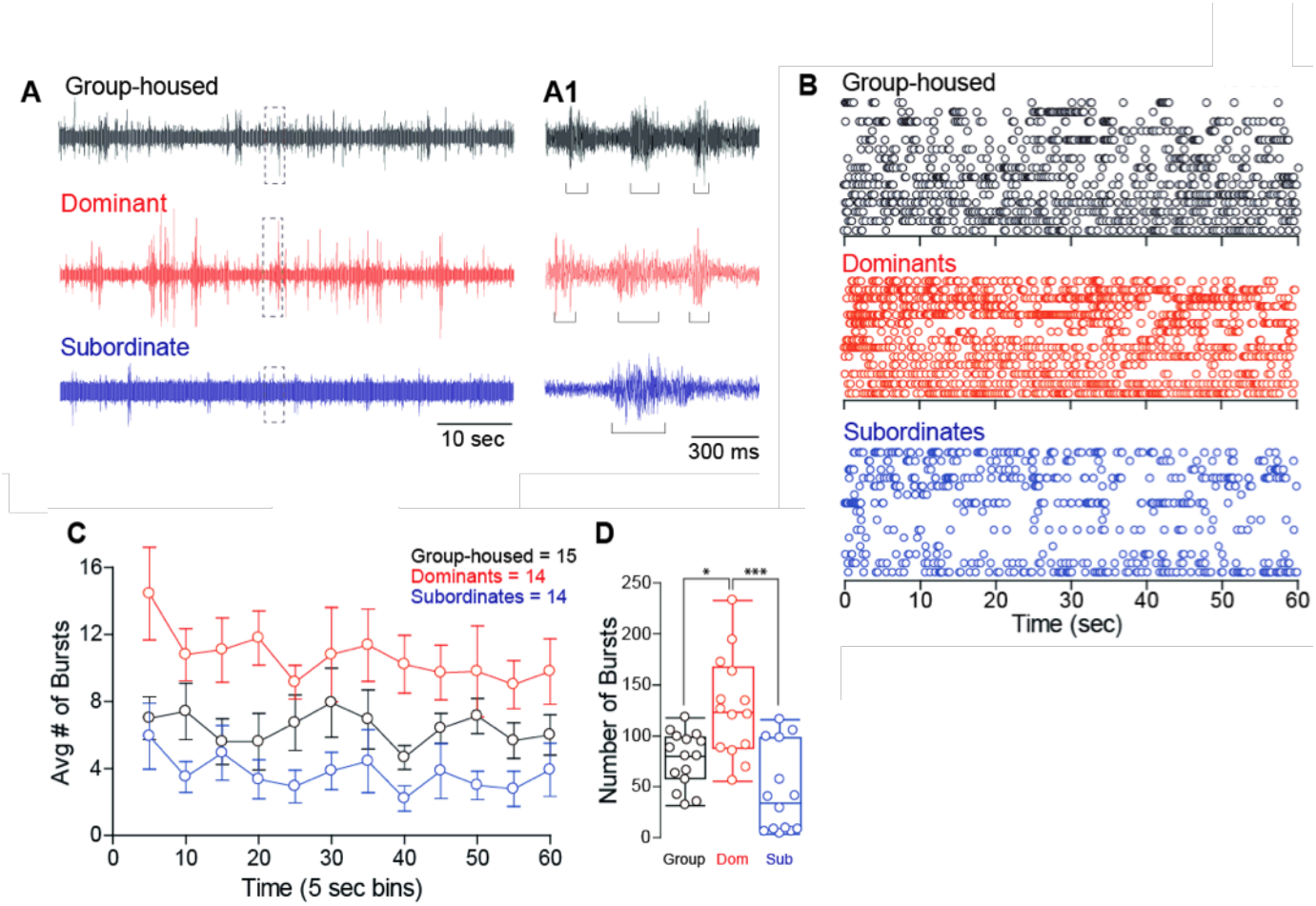

Figure 4 - Social status affects swimming burst frequency

1-minute individual trace recordings of field potentials from group-housed, Dominant and subordinate animals. Swim bursts are easily identifiable and individually sorted (A1, brackets) (dashed boxes denote areas that are enlarged in part A1). B) Raster plots of each social animal group tested. Each row represents the burst swim responses of one animal, and each circle represents one swim burst (see methods for burst analysis and quantification). C) Averaged binned data of swim burst activity of data illustrated in part A and B. (burst activity was binned over 5 seconds). D) Box plot summary illustrating differences in the number of bursts produced over 1 minute of recording. Each dot represents sum of spontaneous swim bursts for each animal during 60 sec of recording. Horizontal line within each boxplot denotes data median, box represents 90% of data. Error bars represent max/min values.

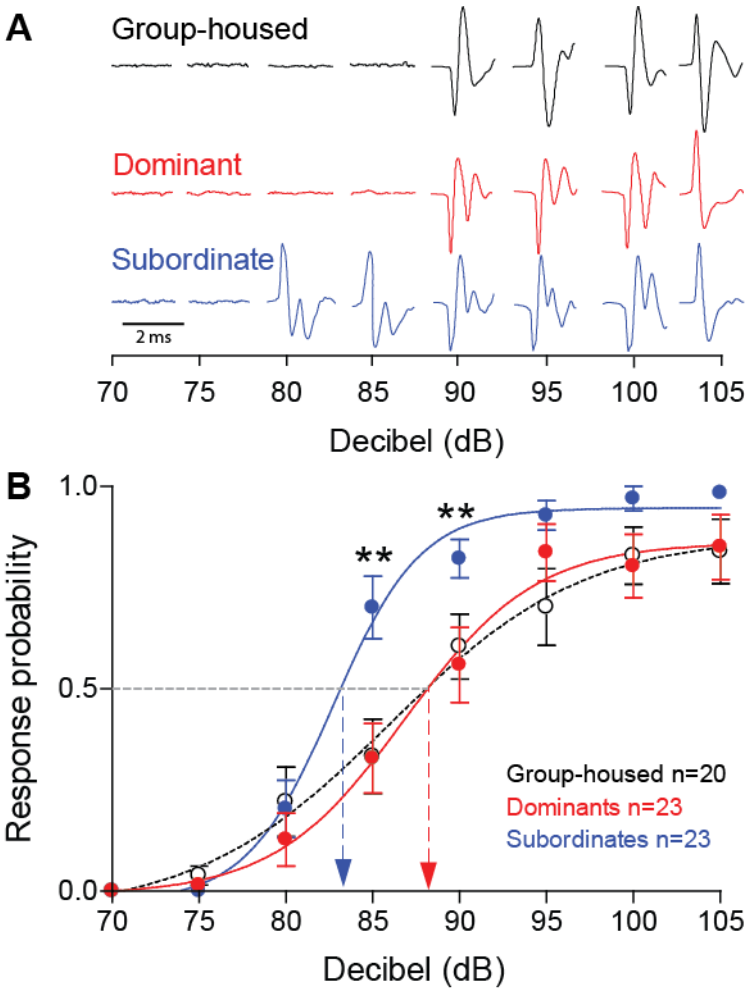

Figure 5 – Social status affects startle escape probability
Startle escape response is significantly more sensitive in subordinate animals compared to dominant and group-housed animals. A) Individual examples of escape field potentials from 3 different animals at increasing decibel intensities. B) Probability of initiating an escape response is significantly higher in subordinates compared to dominants and group-housed animals at 85-90dB. A response probability of 0.5 is indicated with the dotted line

Discussion

Our results showed that male zebrafish formed stable social hierarchies, and that these roles emerged as a consequence of aggressive interactions over a two week pairing period (Figure 3A). We presented data illustrating that as hierarchies were established and stabilized, fish of different social roles preferred specific locations in their housing tank. Subordinates preferred the back corner while dominants swam freely throughout the tank (Figure 3B). We observed significant differences in swimming behavior between dominants and subordinates in their housing tank (Figure 3C). Using non-invasive recording of electric field potentials, we quantified swimming differences between dominant and subordinate fish. By passive recording of swimming in the testing chamber, we observed that subordinates have a decreased swim burst activity compared to group-housed and dominant animals. In contrast, dominants increase their swim burst activity (Figure 4C, D). These results suggested that the underlying neural circuit responsible for swimming behavior may be modified by the social status of the animal.

In addition, this recording technique allowed us to determine how social experience may have modified the M-cell escape response. We presented auditory pulses at varied decibel levels (70-105), and determined that subordinate animals had an increased probability of escape at lower decibels when compared to both dominants and group-housed animals (Figure 5A, B). With these results, it appeared that social status also influenced the sensitivity of the M-cells underlying the escape response. Moreover, our results suggested that activation of neural circuits underlying both swimming and escape behaviors are socially regulated.

To illustrate how social status influenced activation of these two neural circuits, we proposed a simple schematic (Figure 6). The escape circuit is mediated by M-cells that silence the swimming circuit through inhibitory interneurons (i-INs). The swimming circuit is mediated

by central pattern generators (CPGs). Dominants favored activation of the swimming circuit. Subordinates favored activation of the escape circuit and, in turn, inhibited activation of the swimming circuit. In addition to our results, we collaborated with mathematicians to determine what cellular mechanisms account for the observed status-dependent differences in behavior. We built a neurocomputational model (Miller et al., 2017) of the escape and swim circuits based on a simplified representation of the properties of relevant neurons. Overall we were able to mimic empirical results with the computational model by showing that a change in intrinsic synaptic strength between the M-cell and the inhibitory interneurons was sufficient to obtain the transition between dominants and subordinates activity patterns while keeping the network architecture.

With this study, we determined that social experience influenced the activation of neural circuits underlying two behaviors, escape and swim. We showed that dominants increased activation of the swim circuit and showed a lowered activation of the escape circuit, while subordinates increased activation of the escape circuit with a lowered activation of the swim circuit. We modeled our results and observed that the activation of the two circuits can be socially regulated through differences in the synaptic properties of the two circuits. However, the specific synaptic properties underlying these changes in circuit activation still needed to be investigated.

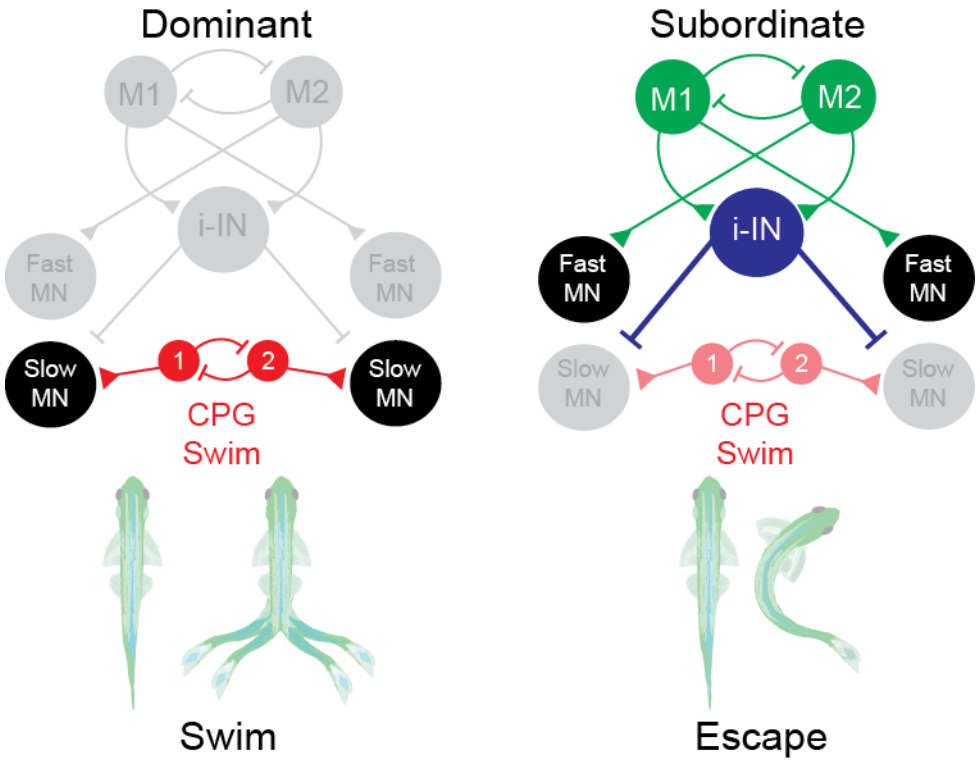

Figure 6 – Proposed schematic illustrating the shift in motor network activation
Our results suggest a shift in activation between escape and swimming in socially dominant and subordinate animals. Suppressed pathways are dimmed, while synaptic pathways that are strengthened are colored emphasized. In dominant animals, low inhibition from inhibitory interneurons with the continuous excitatory input from CPG onto Slow MNs potentiates swimming activity. However, in subordinates, an increase in Mauthner sensitivity promotes the excitation of the escape circuit and inhibition of swim circuit through activation of i-INs.

CHAPTER II

SOCIAL STATUS-DEPENDENT DOPAMINERGIC REGULATION OF TWO NEURAL CIRCUITS

Introduction

In this study we investigated the effects of social status on DA modulation of escape and swim circuits. DA neuromodulation is considered a part of the chemical messenger system. DA is synthesized in the presynaptic neuron and released upon sufficient excitation of that neuron. After release into the synaptic cleft, DA can follow three different fates. It can be recycled back into the presynaptic membrane through a dopamine active transporter (DAT), it can bind to post-synaptic DA receptors or it can be degraded by either monoamine oxidase (MAO) or catechol-O-methyl transferase (COMT).

DA receptors belong to a family of heterotrimeric G-protein coupled receptors (GPCRs). GPCRs consist of seven transmembrane segments with the C-terminal inside the cytoplasm and the N-terminal faced towards the matrix (Yamamoto & Vernier, 2011). There are two classes of DA receptors: D1-like and D2-like. D1-like receptors (D1 and D5) are excitatory. Upon activation, the alpha subunit of the heterotrimeric G-coupled protein exchanges GDP for GTP and binds to the effector protein, adenylyl cyclase (Purves et al. 2012). Activation of excitatory receptors D1 and D5 activates adenylyl cyclase (AC), which causes an excitatory cascade that leads to an active neuron (Purves et al., 2012). In contrast, D2-like receptors (D2, D3, and D4) are inhibitory. When activated, they inhibit adenylyl cyclase, which causes a downstream inhibitory cascade that can lead to eventual decrease in protein phosphorylation.

Both the escape and swimming circuits are regulated by DA (Larson et al., 2006; Oda et al., 1998). In addition, DA is implicated in social regulation and can also influence sensory-

motor programming, such as motivation and aggression (Couppis & Kennedy, 2008; Yamamoto & Vernier, 2011). Previous studies exposed the Mauthner neurons to DA and observed modulation in the sensitivity of the M-cells. Both Pereda et al., (1992) and Pavlik et al., (2005) showed that DA increased the electrical conductivity of mixed synapses connecting sensory input and the M-cells; which increased the probability for the M-cells to fire. In addition to DAs influence on excitability of the M-cells, Jay et al., (2015) ablated supraspinal DA neurons in the zebrafish and observed a reduction in locomotor activity. Application of a D1 antagonist in the region associated with locomotion, the mesencephalic locomotor region (MLR), also decreased locomotor output (Ryczko et al., 2013). With this, we know that DA plays a role in both escape and locomotive circuits.

In addition to the cited literature, Miller and colleagues analyzed the expression of dopaminergic genes in the zebrafish brain and found that there were significant differences in DAT and D1 receptor between social phenotypes (Miller et al., 2016). Dominants showed significantly higher mRNA expression of DAT compared to subordinates, where subordinates showed a significantly lower mRNA expression of D1 receptor. Collectively, these results strongly point to the importance of DA in regulating the escape and swim circuits. This would pose the possibility that the DA system can be socially regulated.

The aim of this study is to determine the importance of the DA receptors in the context of social status-dependent modulation of neural circuit activation through injection of specific DA receptor agonists and antagonists.

Results

The effect of DA supplementation on escape circuit. Augmentation of dopamine through L-DOPA yielded opposite but insignificant effects on the escape response between the two social phenotypes (Figure 7). Dominant animals showed a slight enhancement in escape response probability, while subordinates showed a slight decline in escape response probability (Figure 7A, B). Although the changes in behavior were insignificant, the status-dependent difference between dominant and subordinate phenotype was diminished after injection of L-DOPA (Figure 7C). Overall, the results suggest that DA supply is not responsible for the status-dependent escape differences.

Social status-dependent dopaminergic regulation of escape. To determine the role of the D1 receptor in the escape sensitivity differences between social phenotypes we pharmacologically manipulated D1 receptor activity. After blocking the D1, dominants showed a significant increase in escape sensitivity; particularly with pulses at 80 and 90 dB (Wilcoxon match pairs t-test, p=0.0156 at 80dB, P=0.0313 at 85dB; Figure 8A). Subordinates, however, did not show any behavioral change (Figure 8B). Comparison of dominants' response after blocking the D1 receptor to subordinates' showed a shift in escape sensitivity of dominants to reflect that of subordinates (Figure 8C). After activating the D1 receptor, dominants decreased their escape sensitivity, where subordinates did not show any behavioral change (Figure 8D & E). Neither blocking nor activating the D2 receptor showed significant changes in escape probability with dominants or subordinates (Figure 9). The D3 receptor showed no effect on dominants. However, subordinates showed a significantly lowered probability of escape after blocking the D3 receptor (Wilcoxon match pairs t-test, P=0.0039 at 80dB; Figure 10). These results suggest that the differences in escape behavior in dominant and subordinate animals may be influenced

by activation of different dopaminergic receptors.

L-DOPA

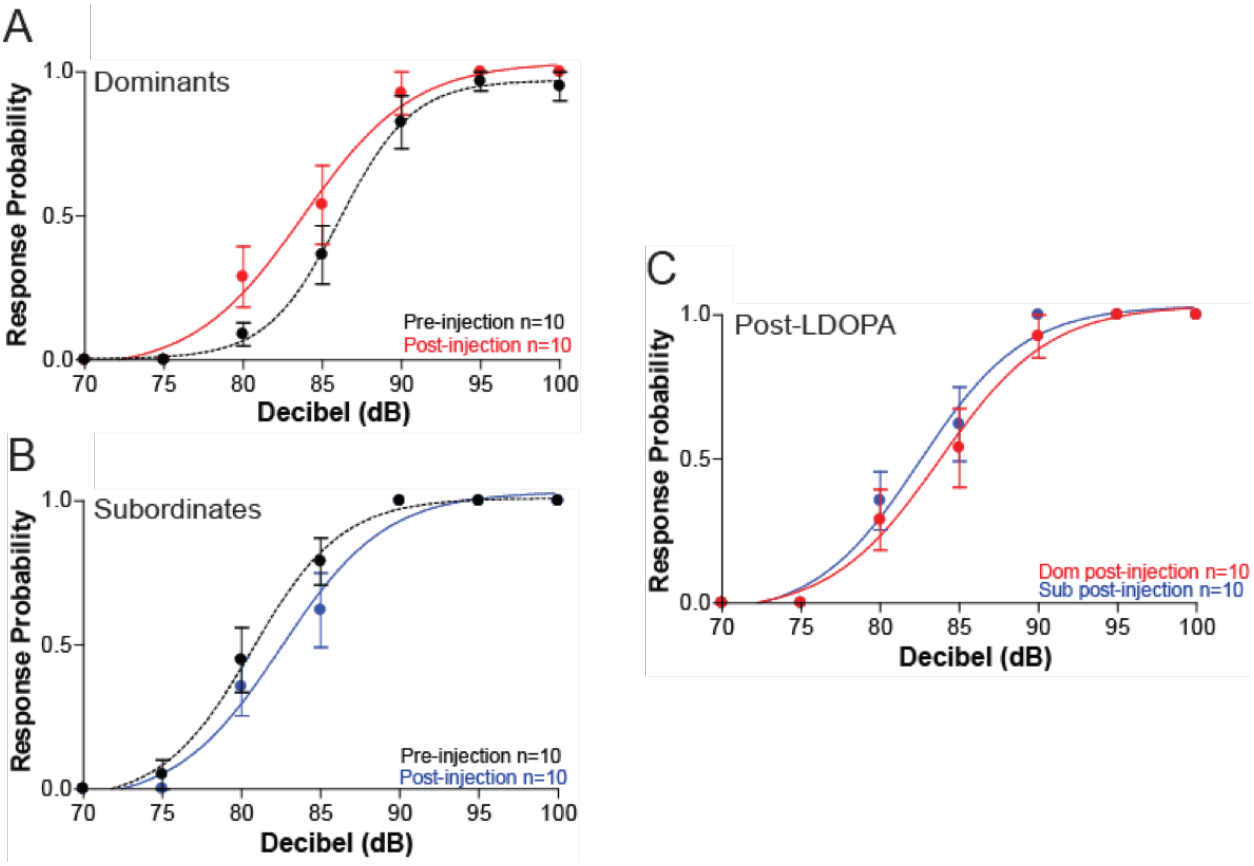

Figure 7 – Augmentation of dopamine on escape sensitivity

Augmentation of dopamine affects the escape sensitivity of both social phenotypes. A) Dominants (red) show a slight shift towards a higher escape response probability, while subordinates (blue) show a slight shift towards a lower probability B). C) comparison of dominant (red) and subordinate (blue) escape probability after injection. Significant differences that are seen in phenotypes naturally are lost after augmentation of dopamine.

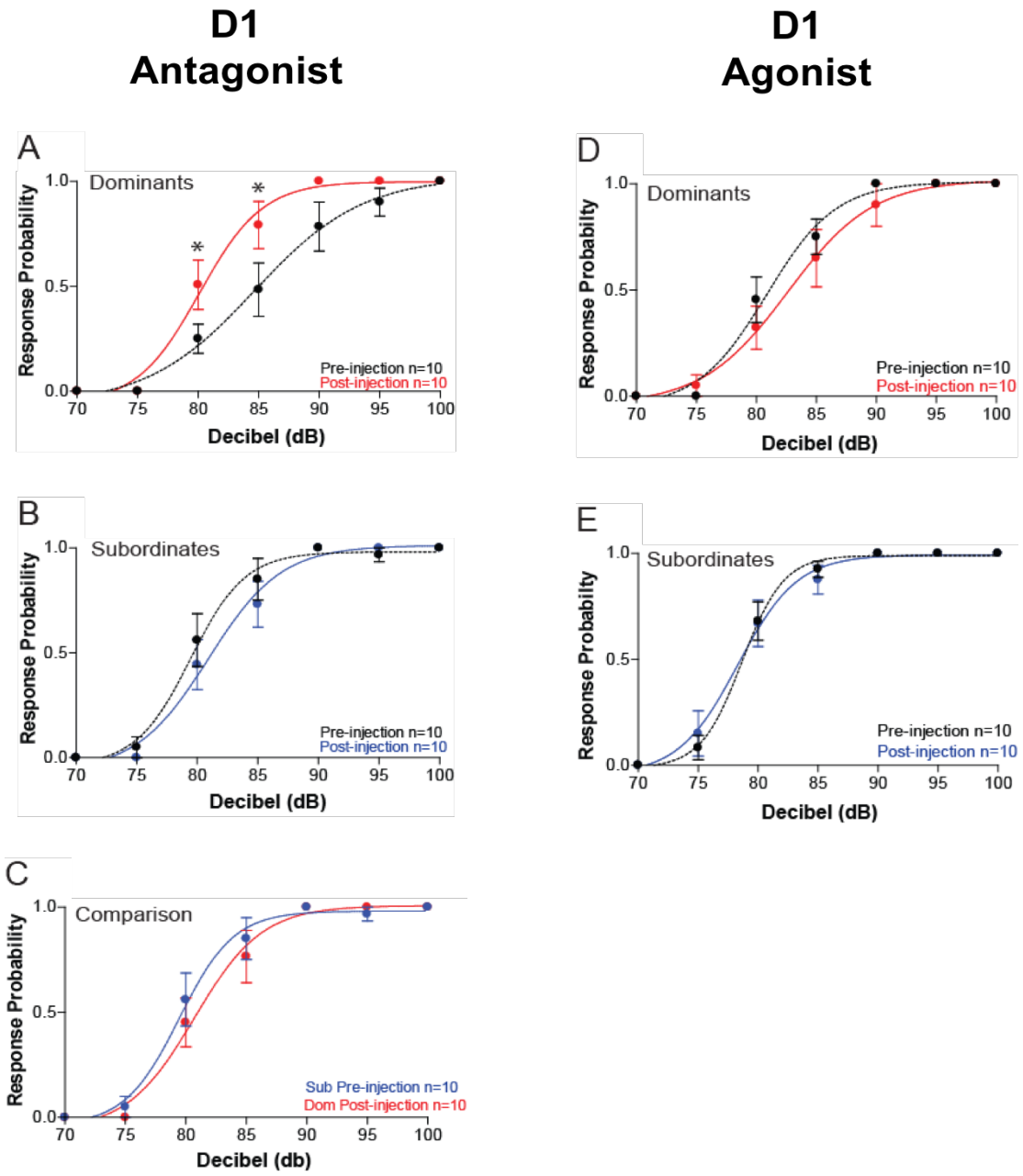

Figure 8 – D1 receptor influence on escape behavior

Pharmacological manipulation of D1 receptor activity. After blocking the D1 receptor with SCH23390, dominants showed a significantly increased probability for escape (A), where subordinates showed no effects (B). When compared, dominants shift their escape behavior to reflect subordinates (C). (D, E) Activation of the D1 receptor with Dihydrexidine yielded no changes in behavior.

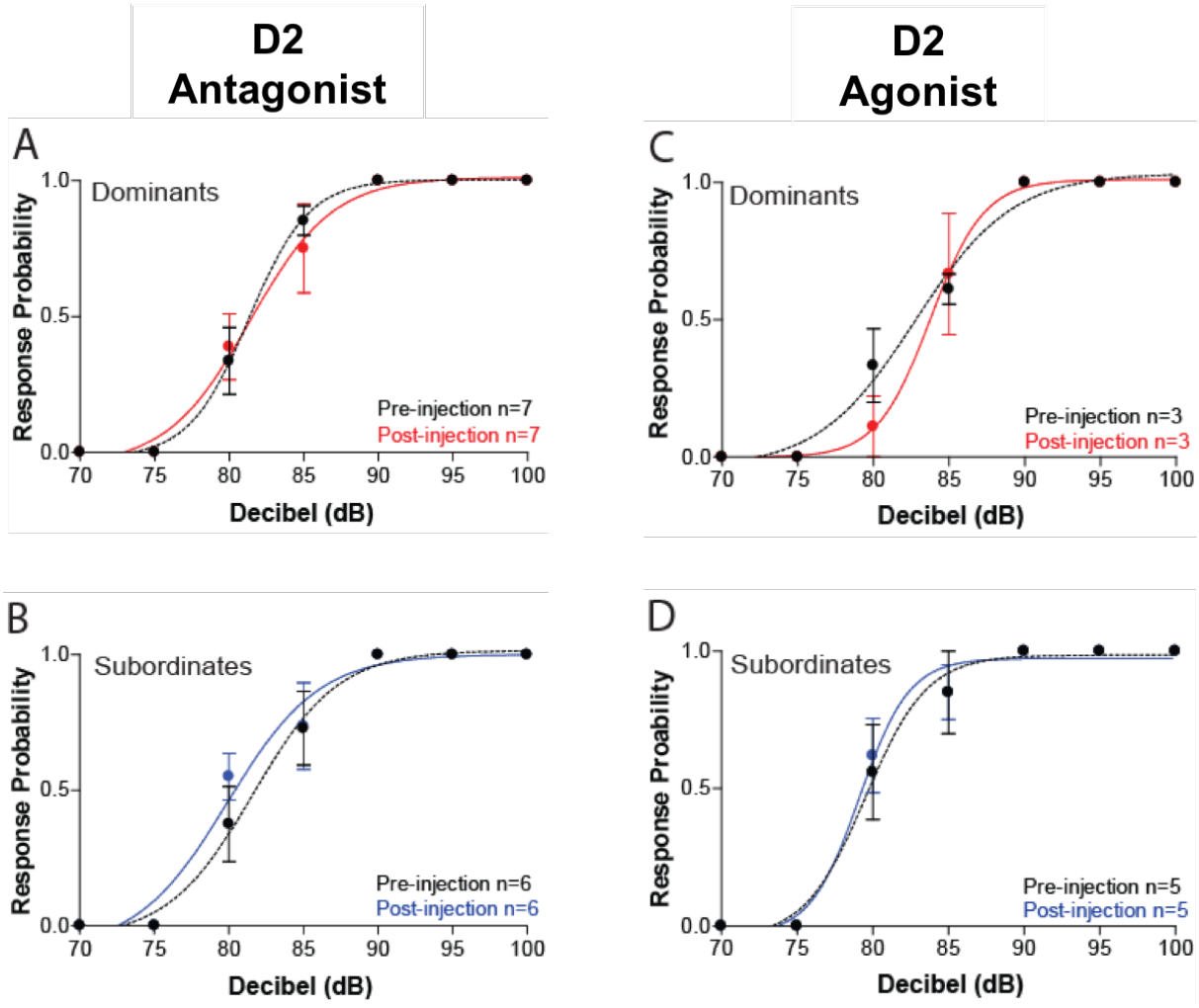

Figure 9 – D2 receptor influence on escape behavior

There were no effects on escape response probability of either social phenotype after blocking or activating the D2 receptor. (A & B) Neither dominants nor subordinates show a change in escape behavior after blocking the D2 receptor with Raclopride. Similarly, activating the D2 receptor with Bromocriptine yielded no changes in escape or swim in dominants (C) or subordinates (D).

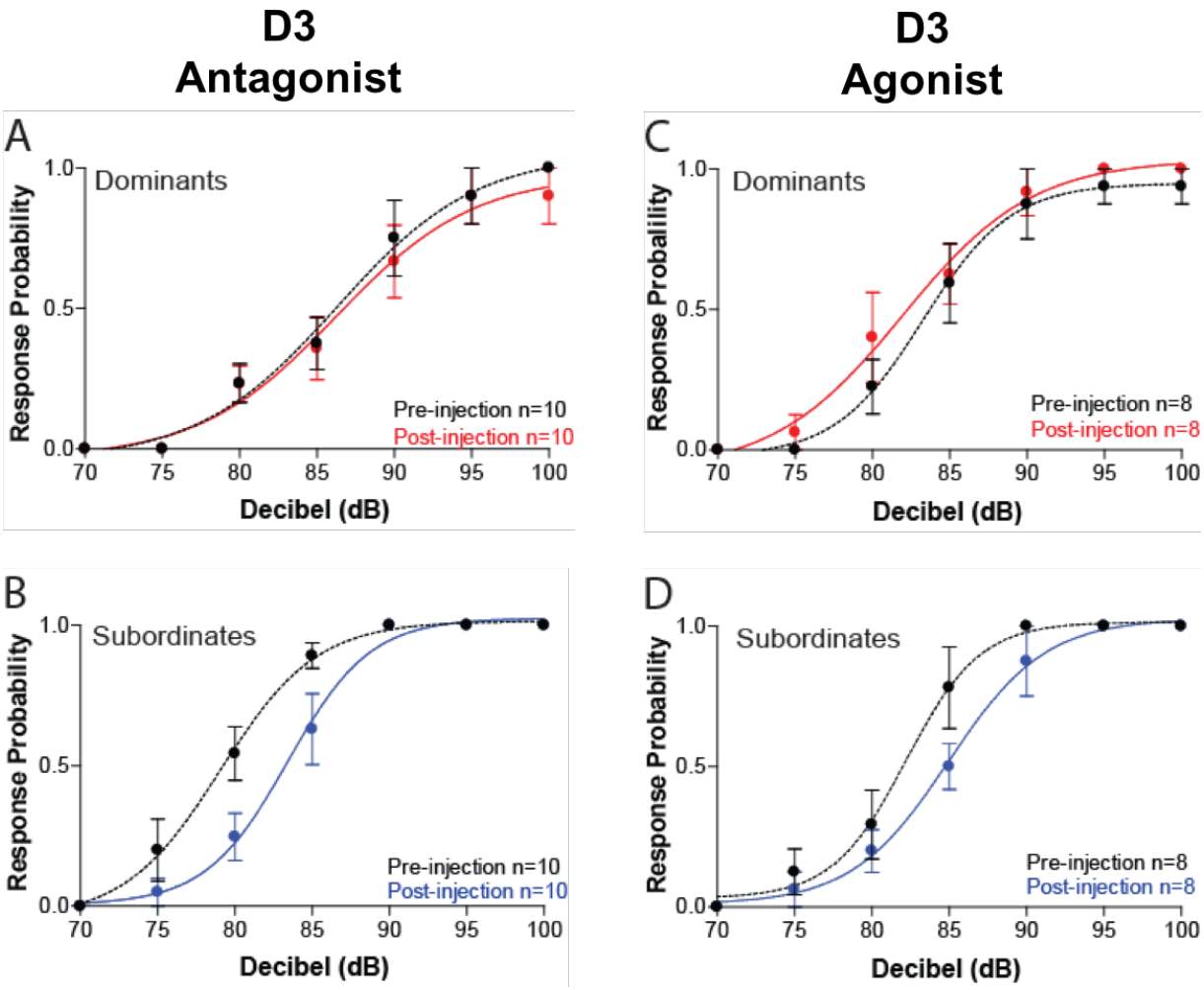

Figure 10 – D3 receptor influence on escape behavior

Pharmacological manipulation of D3 receptor activity. Blocking the D3 receptor with SB 277011A yielded no changes in dominants (A), however, subordinates showed a significant decrease in escape probability (B). (C, D) Activating the D3 receptor with Pramiprexole yielded no behavioral changes in either dominants or subordinates.

Social status-dependent dopaminergic regulation of swimming. In addition to escape, we were interested in whether the status-dependent differences in swimming activity we documented in chapter 1 were caused by DA modulation (Figure 4). Increasing DA levels though L-DOPA did not alter swimming behavior in neither dominants nor subordinates (Figure 11). After blocking the D1 receptor, dominants swimming behavior significantly decreased compared to their pre-injected swimming behavior (Figure 12A, top). In contrast, blocking the D1 receptor caused no effect on subordinate swimming (Figure 12A, middle). Importantly, the decrease in swimming activity in dominants due to blocking D1 receptor activity reached similar levels to those of subordinates natural swimming (Figure 12A, bottom). Although the D2 receptor does not appear to be involved in the social status-dependent regulation of the Mauthner escape response, pharmacological manipulation of its activity yielded status-dependent changes in swimming behavior. Blocking the D2 receptor with the antagonist Raclopride, caused a significant downward shift in dominant swimming behavior (Figure 13A, top, Wilcoxon match pairs t-test, P=.0020). However, subordinates did not show a change in their swimming behavior (Figure 13A, bottom). Activation of the D2 receptor with the D2 agonist, Bromocriptine, yielded no change in either dominant or subordinate animals (Figure 13B). Antagonizing the D3 receptor with SB 277011A yielded no effects on swimming in either social phenotype (Figure 14A, B). However, after activating the D3 receptor with Pramiprexole, dominants swimming significantly decreased (Figure 14C, Wilcoxon match pairs t-test, P=0.0371) and there was no change in subordinate swimming (Figure 14D). These results suggest that both the D1/D3 receptors seem to be molecular regulators for the differential activation of escape and swim behaviors between dominant and subordinate fish. The D2 results stand out in that although there is seemingly no status-dependent regulation on the escape circuit, the results suggest that D2 receptor may play a

prominent role in differentially regulating the swimming circuit in a socially dependent manner.

L-DOPA

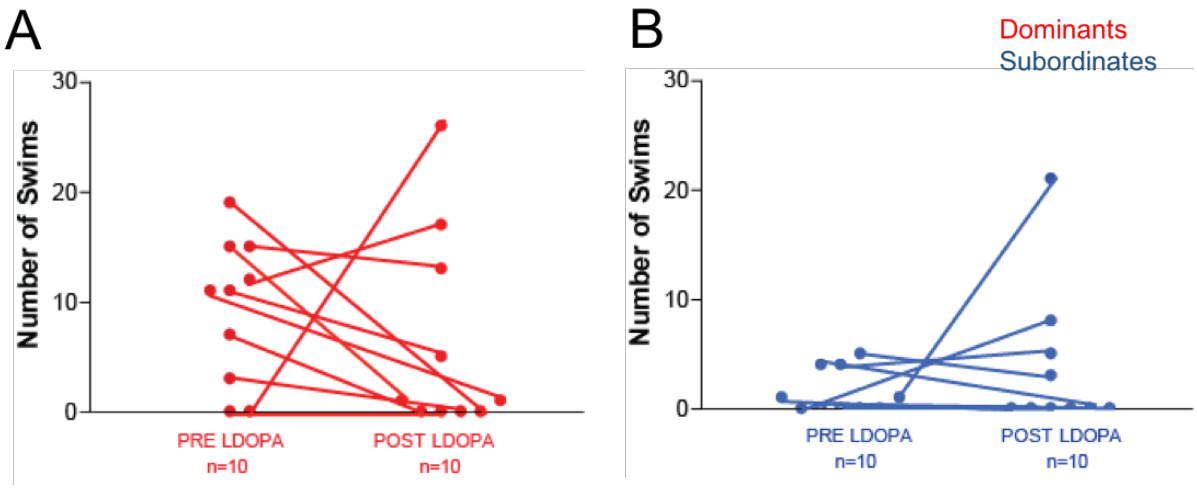

Figure 11 - Augmentation of DA on swimming behavior

After augmentation of DA, swimming behavior was not significantly changed in either dominants (A) or subordinates (B).

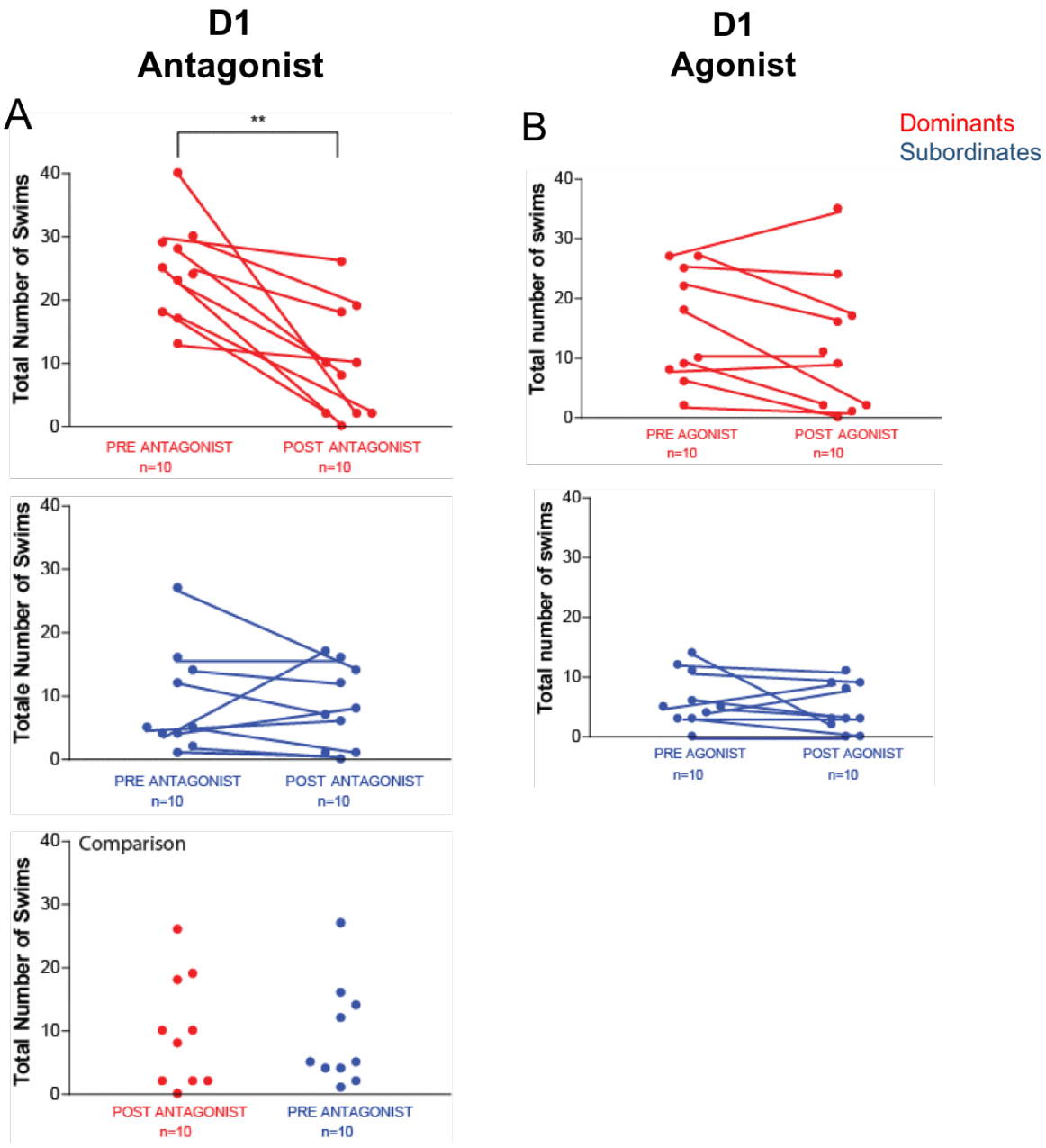

Figure 12 – D1 receptor influence on swimming behavior

Blocking the D1 receptor with SCH23390 led to a significant decrease in dominant swimming behavior (A, top), while having no effect on subordinates (A, middle). When compared, dominant swimming behavior after blocking the D1 receptor resembles subordinate swimming behavior (A, bottom). Activating this receptor with Dihydrexidine yielded no changes in swimming with dominants or subordinates (B).

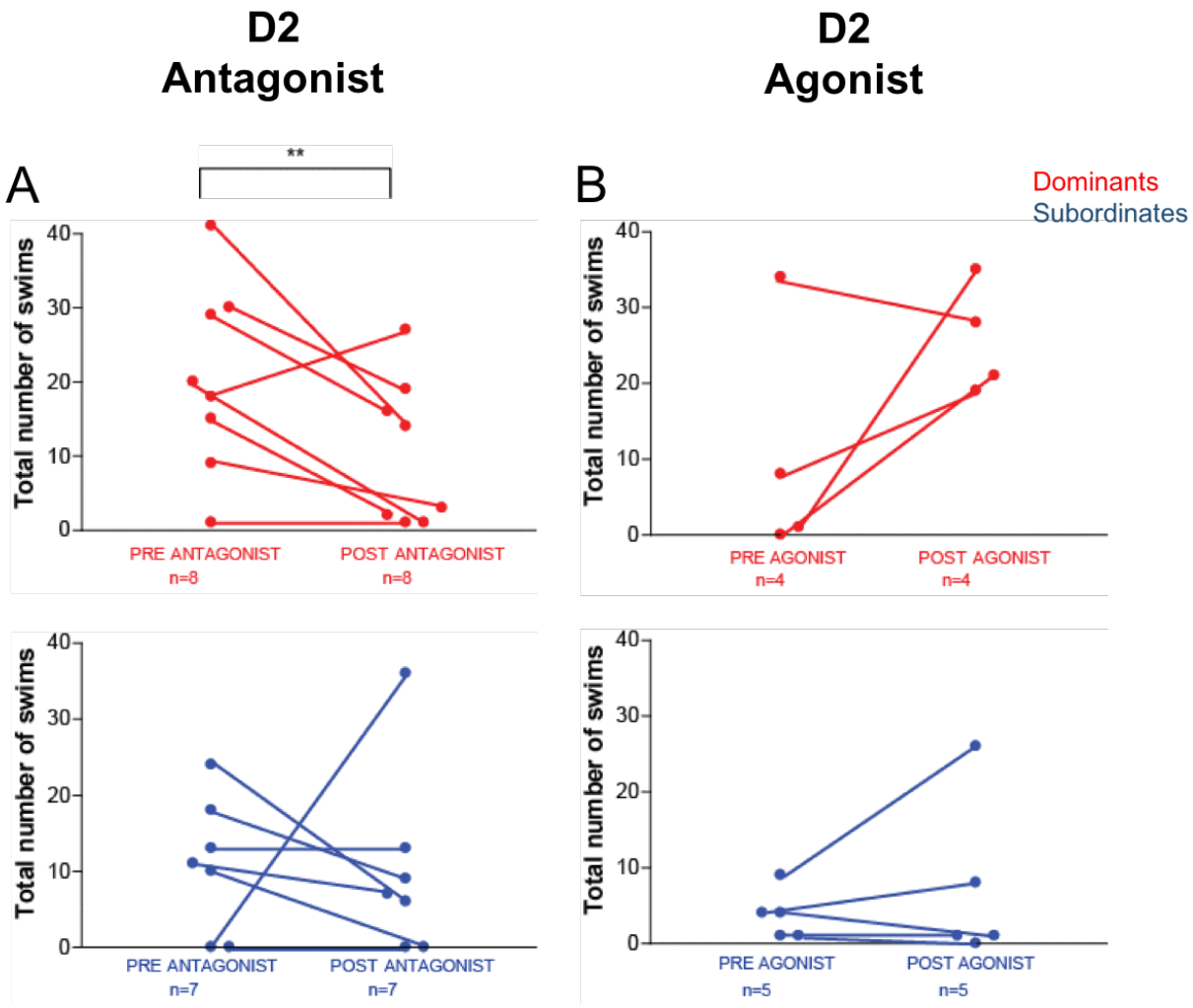

Figure 13 – D2 receptor influence on swimming behavior

Blocking the D2 receptor with Raclopride caused a significant decrease in dominant swimming behavior (A, top), with no changes in subordinates (A, bottom). Activating the D2 receptor with Bromocriptine yielded no changes in either dominants or subordinates (B).

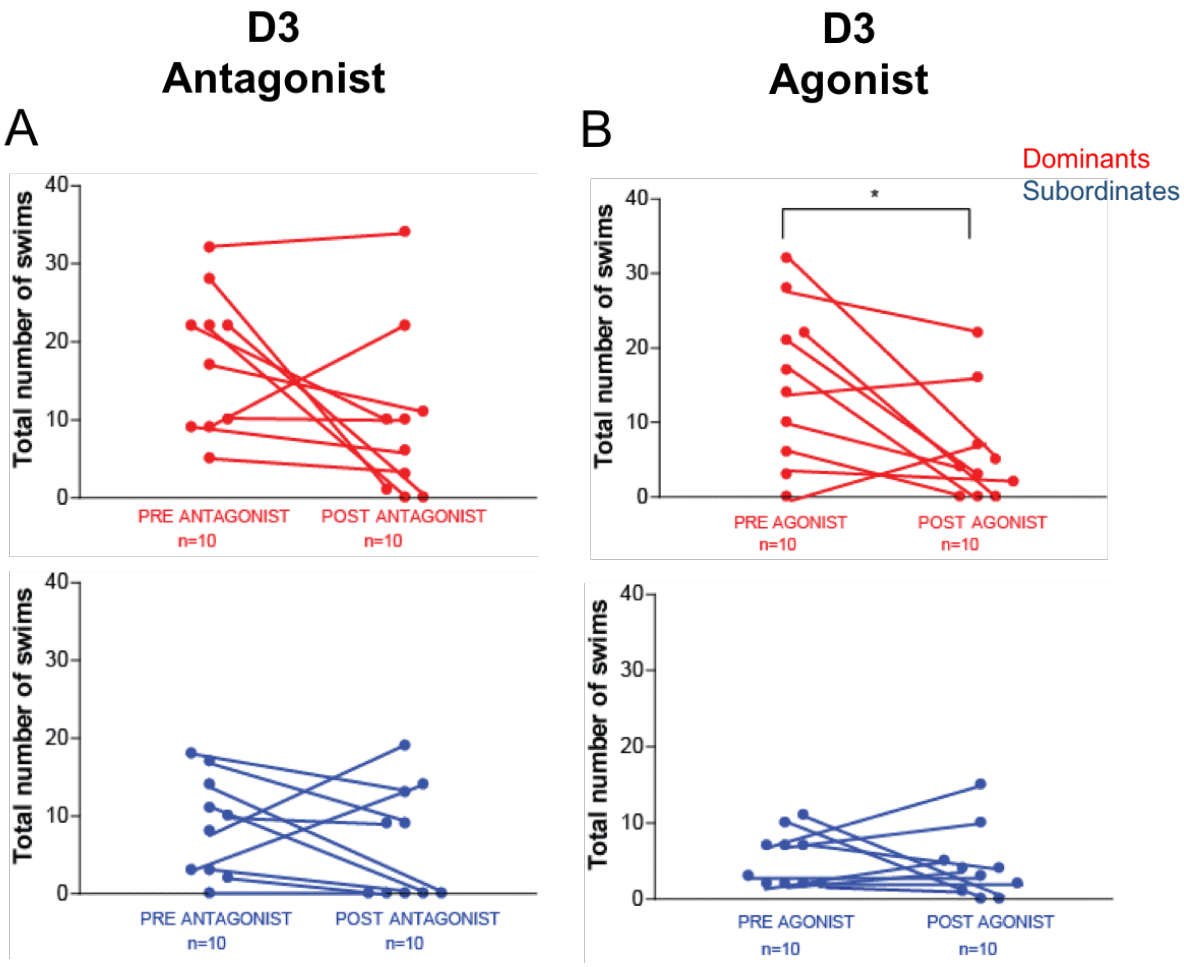

Figure 14 – D3 receptor influence on swimming behavior

The D3 receptor differentially affects swimming based on social status. Blocking the D3 receptor with SB 277011A yielded no changes in dominants (A, top) or subordinates (A, bottom). Activating the D3 receptor with Pramiprexole caused a significant downward shift in swimming behavior in dominants (B, top) with no changes in subordinates (B, bottom).

Discussion

Zebrafish formed stable social hierarchies that consisted of dominant and subordinate animals, and these hierarchies influenced whether the animal favored activation of escape or swim behavior. It is known that underlying neural circuits responsible for both behaviors are influenced by dopaminergic modulation (McLean & Fetcho, 2004; Oda et al., 1998) and that DA signaling is a determinant for aggressive behavior (Filby et al., 2010). However, whether DA regulates these two behavioral circuits in a social status-dependent manner has yet to be fully explored. Here we show that augmentation of DA yielded differential, but insignificant, results for dominants and subordinate escape and swimming behaviors (Figure 7 & 11). These results suggested that if DA is involved in the status-dependent differences in swimming and escape behaviors, it may be occurring post-synaptically with the receptors.

Miller et al. (2016) performed whole brain gene expression analysis on genes involved in dopaminergic signaling and proposed two potential targets for social status-dependent differences: dopamine reuptake transporter (DAT) and the D1 receptor. The authors discovered that dominant animals significantly upregulated DAT gene expression compared to subordinates, while subordinate animals significantly downregulated the D1 receptor gene expression compared to dominants. DA receptors have been previously implicated in the involvement of social hierarchies (Watanabe & Yamamoto, 2015; Yamaguchi et al., 2016; Yamaguchi et al., 2017); therefore, we decided to focus on the interpretation of DA signaling by pharmacologically manipulating DA receptor activation through systemic injection of DA specific agonists and antagonists.

The D1 receptor is classified as an excitatory receptor for its amplification of adenylyl cyclase (Purves et al., 2012). After blocking the D1 receptor in dominants, there was a

significant enhancement of their escape response, resembling subordinate escape sensitivity. Conversely, after activating this receptor, dominants displayed a lowered escape sensitivity typical of dominant animals (Figure 8A & D). Subordinate animals did not show change in escape probability after blocking or activating the D1 receptor (Figure 8B & E). These results suggested that the D1 receptor is involved in modulating the sensitivity of the M-cells and supported the gene expression data that showed subordinate downregulation the D1 receptor (Miller et al., 2016). Interestingly, these results are contradictory to what would be expected when blocking an excitatory receptor. Blocking an excitatory receptor should yield inhibition. Our results suggest that DA may be influencing the M-cells indirectly through an inhibitory pathway (see general discussion for details).

In addition to escape, after blocking the D1 receptor, dominants showed a significant decrease in their swimming frequency, resembling a subordinate phenotype (Figure 12). Activation of the D1 receptor in dominants yielded no difference in swimming behavior compared to pre-tests. On the other hand, subordinates showed no changes in swimming frequency after blocking or activating the D1 receptor (Figure 12). These results suggested that D1 receptor also regulates the swimming circuit in a social status-dependent manner. Taken together, it appears that the D1 receptor may be a molecular regulator for circuit activation dynamics between swimming and escape behaviors. Moreover, the activation of the D1 receptor within these two circuits seems to be modulated by social rank.

Our results suggested that the D2 receptor is not a primary player in the social status-dependent excitability of the M-cell. Neither blocking nor activating this receptor yielded behavioral differences in escape behavior for dominants or subordinates (Figure 9). However, this could be due to a small sampling size. In addition, further confirmation via single cell RT-

PCR or quantitative immunohistochemical assay of the M-cell should be performed to verify these results. These added experimental approaches would allow us to determine whether the D2 receptor is expressed on the M-cell and would also provide visual confirmation of its presence or absence. From the previous chapter it appeared that the activation of these two circuits can depend on each other for the behavioral output of an animal; however, they are distinct circuits. Although our results suggested that the D2 receptor may not be involved in modifying status-dependent escape behavior, we observed that blocking the D2 receptor caused dominants to significantly decrease swimming frequency (Figure 13A, top). Since our injection protocol is a systemic injection, the behavioral changes might be the result of a blockage of D2 autoreceptors on an inhibitory pathway within the spinal cord, leading to continued inhibition of spinal cord motor neurons.

Miller and colleagues reported that whole brain gene expression of the D3 receptor did not differ between dominants and subordinates (Miller et al., 2016). However, our pharmacological results showed significant differences in how D3 affects the escape and swim circuits in dominant and subordinate animals. Dominants did not exhibit behavioral changes when the D3 receptor was blocked or activated; however, subordinates became significantly less likely to produce an escape (Figure 10B). The D3 receptor is categorized under D2-like receptors in that they inhibit adenylyl cyclase (Purves et al., 2012). If the D3 receptors are directly influencing the M-cells, we would expect that blocking these receptors would lead to an increase in escape response probability. However, this was not the case. By blocking the D3 receptor and observing that subordinates show a decreased response probability, these results also suggest that the DA receptors are influencing the M-cells through an inhibitory pathway. Although dominant escape probability was not affected by blockage or activation of the D3 receptor, activating D3

caused a significant decrease in swimming frequency (Figure 14A, top). These results suggest that activation of the D3 receptor may also be involved in the status-dependent differences in escape and swimming behaviors.

When administering receptor agonists and antagonists, it is important to use a ligand with the highest affinity for the targeted receptor. The D1 receptor agonist used in this study, Dihydrexidine, has only a 10-fold selectivity for D1-like receptors over D2-like receptors (Mu et al., 2007). A better substitute for this agonist would be SKF-81297, which has a 200-fold selectivity for D1 over any other receptor (Zhang et al., 2009). The D2 receptor agonist used, Bromocriptine, also has a high affinity for the D3 receptor (Kvernmo et al., 2006). In addition, Tan & Jankovic (2001) reported that this drug has D1 receptor antagonistic properties. To confirm the lack of significance seen with our D2 agonist, we need to administer another drug with a higher selectivity of the D2 receptor over the D3 receptor that also lacks any additional dopamine receptor antagonist properties.

Overall, with this study we gained a better understanding of DAs role within social status-dependent changes underlying swimming and escape circuits. It appears that the escape circuit is primarily being modified through D1/D3 receptor activity. To a lesser extent, it appears that the D1, D2, and D3 receptors are involved in regulating swimming circuit in a status-dependent dependent manner. Our results raise more exciting questions. Which inter-neuronal pathway might DA be influencing the escape response? What is the relationship between D1/D3 on this pathway? Would other mechanisms, such as degradation or cell excitability contribute to these behavioral changes? Finally, how do we tease apart the contributions of each of these receptors on swimming activity? These questions will be further addressed and explored in the next chapter.

CHAPTER IV

GENERAL DISCUSSION

Social hierarchies are important for stability of social communities and are usually formed through aggressive interactions. A stable social relationship is formed between paired animals and a dominant and subordinate animal emerge. How do social hierarchies influence underlying neural circuits responsible for behavioral output? Using zebrafish as a model, we studied how stable social hierarchies influenced swimming and escape behavior of animals occupying different social status. Subsequently, we focused on the role of DA in modulating the circuits underlying these two behaviors. DA is known to be involved in social regulation, aggression, and hierarchy formation (Larson et al., 2006; Oliveira et al., 2011; Pavlidis et al., 2011). This motivated us to determine whether DA is involved in the status-dependent regulation of escape and swim behaviors.

Our experiments revealed that zebrafish formed stable social hierarchies as a result of aggressive/submissive interactions (Figure 3) and that the rank of the fish influenced its probability of escape and swimming. Dominant fish increased their swimming frequency over escape, while subordinates increased escape probability over swimming frequency (Figures 4 & 5). To determine whether the supply of DA was involved in the status-dependent differences between the two circuits underlying these behaviors, we attempted to augment DA in both dominants and subordinates through injection of L-DOPA. We found that augmenting DA levels via L-DOPA injections did not alter the animals' escape sensitivity or swimming frequency (Figure 7 & 11). These results suggested that DA levels had no direct effect on these behaviors. However, further experiments are needed to confirm this notion by performing High Precision Liquid Chromatography (HPLC) as laid out in Chatterjee & Gerlai (2009) to confirm that levels of DA were indeed increased after administration of L-DOPA. Miller et al. (2016) analyzed

whole-brain mRNA expression levels of genes within DA signaling and determined that dominants significantly upregulated DAT mRNA compared to subordinates. Conversely, subordinates significantly downregulated the D1 receptor mRNA compared to dominants. We tested the roles of the DA receptors in status-dependent behavioral changes through injection of agonists and antagonists. We found that by blocking the D1 receptor, dominants shifted both escape probability and swimming frequency to a subordinate phenotype (Figure 8 & 12). In addition, we found that by blocking the D3 receptor, subordinates shifted their escape probability to reflect a dominant phenotype (Figure 10). Additionally, blocking the D3 receptor caused dominants to decrease their swimming frequency (Figure 14). No significance was observed in escape probability of either social phenotype after blocking or activating the D2 receptor; however, blocking D2 resulted in dominants decreasing their swimming behavior (Figures 9 & 13). Taken together, the significant results from this study raise new questions regarding where these receptors are located and how they may be working synergistically to produce the observed behavioral effects from the administration of agonists and antagonists.

The results from administration of the D1 and D3 receptor antagonists suggested that these receptors might be indirectly influencing the M-cell escape response through activation of an i-IN. If the excitatory D1 receptor is located on the M-cells, blockage of this receptor would yield a lower escape response probability. However, when we blocked the D1 receptor, dominants displayed excitation in their escape response, resulting in a higher escape probability. In addition, results from Miller et al., 2016 suggested that subordinates presumably expressed a lower number of D1 receptors. Our behavioral results showed that subordinates displayed an increased excitation of the escape response and a higher escape probability. If the inhibitory D3 receptor is located on the M-cells, blockage of this receptor would yield excitation and a higher

escape probability. However, subordinates shifted their escape probability to a dominant phenotype when we blocked the D3 receptor. If the D1/D3 receptors were located on an i-IN, which then influenced M-cell activation, the observed results would not conflict with our understanding of the mechanisms behind excitatory and inhibitory receptors. To build off of the circuit we proposed in chapter one (Figure 6), there are two different possibilities of where the potential i-IN may be located. The first is a method of feed-back inhibition from the i-IN located between and acting on both the escape and swim circuits. However, there could also be a second, independent i-IN from another brain nucleus that influences only M-cell excitability. These two possibilities are illustrated below in Figure 15.

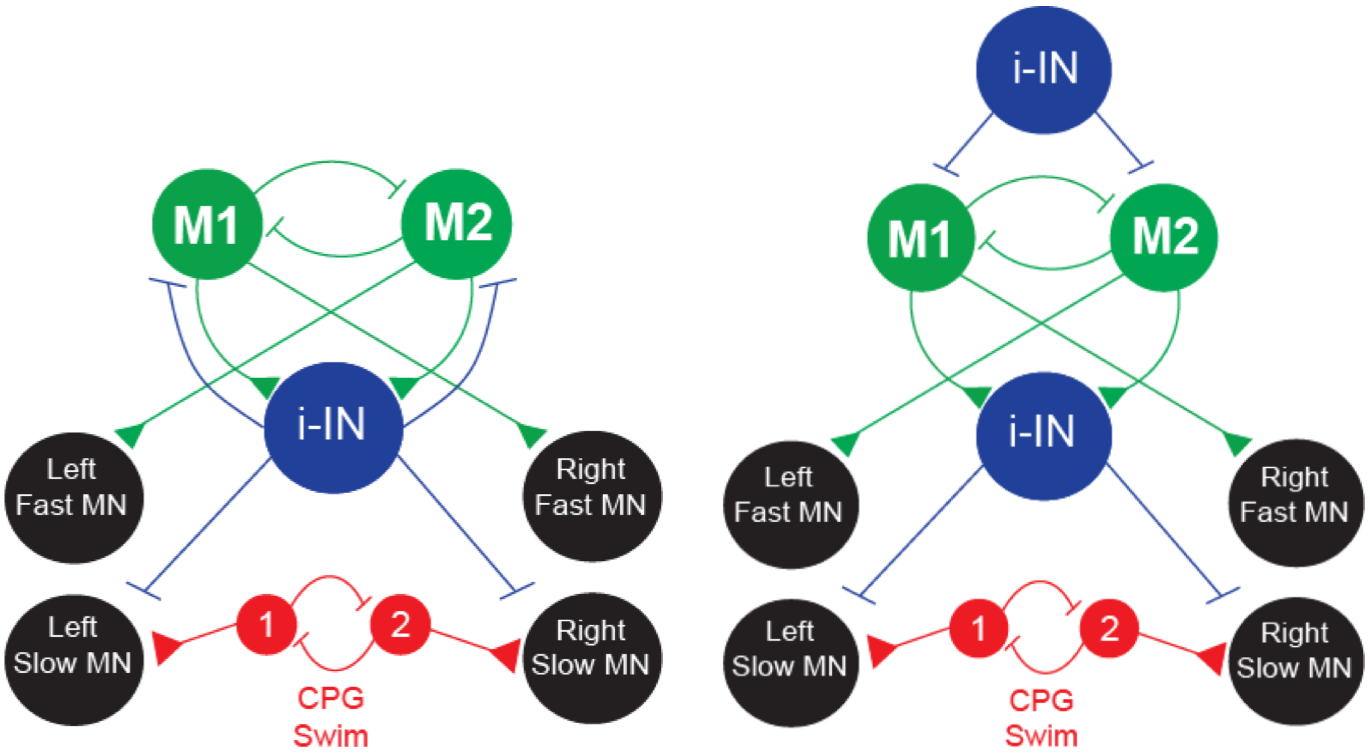

Figure 15 – Two model circuits of possible mechanisms of inhibitory influence on the M-cells

Our D1/D3 antagonist results suggested that the receptors may be located on an i-IN. The first possible location of the i-IN (Shown on the left) is within the proposed circuit from chapter one, where feedback inhibition also influences M-cell excitability. The second possible location (Shown on the right) is that the i-IN is located in a brain nucleus independent of the i-IN in the proposed model and acts only on the M-cells.

In regard to swimming behavior, blocking the D1 receptor decreased dominant swimming frequency (Figure 12A, top). This result suggested that the D1 receptors might be located on the i-IN located within a separate brain nucleus that acts separately on the M-cells (Figure 15, right). In addition, if the feed-back i-IN was the correct model circuit (Figure 15, left), we would expect to see a higher escape probability and an increased swimming frequency from blocking the D1 receptor. However, what we observed was that blocking the D1 receptor caused dominants to increase their escape probability while simultaneously decreasing their swimming frequency. This supports the notion that there may be two separate inhibitory inputs; one primarily influencing the M-cells and another influencing the switch between escape and swimming behaviors.

Additionally, we observed that blocking D3 receptors caused a decrease in subordinate escape frequency (Figure 10B). If the D3 receptors were located on the i-IN that is responsible for feed-back inhibition onto the slow MNs and simultaneous feedback inhibition onto the M-cells, then blocking these receptors would lead to an increased escape probability and a decreased swimming frequency. However, what we observed was that blocking the D3 receptor led to a decrease in subordinate escape probability with no change in swimming frequency. Notably, subordinates displayed a low swimming frequency, so the lack of decreased swimming frequency could be attributed to a basement effect. Interestingly, when the D3 receptor was activated, dominants significantly decreased their swimming frequency (Figure 14B), but there was no change in escape probability. These results may be a result of the D3 receptor acting on receptors within the swim circuit of dominant animals independently of the connection between the escape and swim circuits.

Although subordinates resembled dominant escape probability after blocking the D3,

gene expression analysis from Miller et al., (2016) did not show a decreased level of D3 mRNA in dominants. Additionally, by activating the D3 receptor, we did not see an increased escape probability. This raises the question of how these receptors are working together to produce the escape probabilities observed in dominants and subordinates. Our data suggested that social status affected how both D1 and D3 receptors influenced escape and swimming behaviors. Both the D1 and D3 receptors co-localize in peripheral and nervous system tissues (Brewer et al., 2014; Sidhu & Niznik, 2000; Maggio et al., 2015; Fiorentini et al., 2008, Zeng et al., 2004). Current research revealed that GCPRs can exist as homodimer, heterodimers, and heterotrimers (Jordan & Devi, 1999; Fiorentini et al., 2008; Ferre et al., 2014). Fiorentini et al. (2008) described in length that the D1/D3 receptors form heterodimers and how this complex affects the functional regulation of the D1 receptor. Although the D3 receptor naturally has a higher affinity for binding with DA (Levant, 1997), the formation of a heterodimer can shift the affinity to the D1 receptor and subsequently potentiate adenylyl cyclase (Fiorentini et al., 2008).

If there is a D1/D3 heterodimerization occurring on the i-IN that influences the M-cell, we would expect that the effects of DA would potentiate D1 signaling; activating the i-IN and increasing inhibition on the M-cells. In turn, the i-IN would inhibit the M-cell from firing and we would get a lowered escape probability, resembling dominant behavior. In addition, if the D1 receptor is downregulated, this would cause a loss in heterodimerization and more independent D3 receptors. These receptors, now having the higher affinity, cause the i-IN to be inhibited, and removal of net inhibition on the M-cells. As a result, we would expect to see a higher escape probability, reflecting subordinate behavior. Miller et al. (2016) saw a significant downregulation of the D1 receptor in subordinates, and behavioral testing showed that subordinates escape with a higher probability than dominants and group-housed animals (Figure

5). We have also seen that by blocking the D1 receptor, dominant escape probability is significantly heightened, resembling subordinates (Figure 8). Taken together, it is possible that these receptors may be influencing M-cell sensitivity by forming a heterodimer complex on the hypothetical i-IN. In addition to escape, blocking the D1 receptor causes dominants to significantly reduce swimming frequency; reflecting subordinate swimming. These behavioral results mimic what we would expect with subordinates if there is a D1/D3 heterodimerization on an i-IN regulating the M-cells. Subordinates downregulate the D1 receptor, leaving D3 to inhibit the i-IN. As a result, the inhibition of the M-cells is removed, allowing for more excitable M-cells and a higher escape probability.

To illustrate how this downregulation in the D1 receptor causes a loss in the D1 favored signaling heterodimerization and an increased D3 favored activation in subordinates, we built on our proposed model (Figure 16). This schematic lays the groundwork for future experiments to determine the location of receptor expression as well as if this heterodimer between the D1 and D3 receptors is occurring on the i-IN.

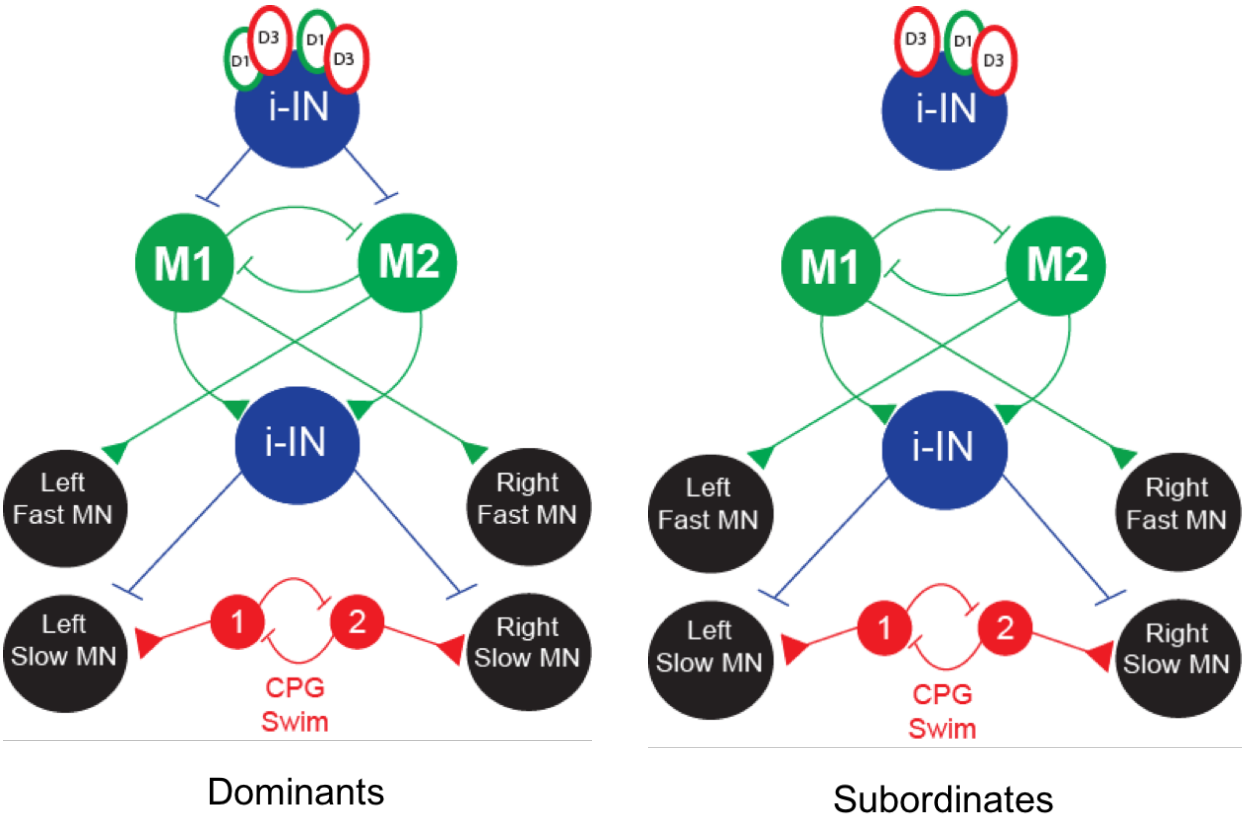

Figure 16 - Proposed model of DA receptor heterodimerization influence M-cell excitability

Our proposed model illustrates the D1/D3 heterodimerization and downstream effects on M-cell excitability. The heterodimer favors activation of the D1 receptor, which allows for the i-IN to inhibit the M-cells from firing. This event is reflected in dominants (shown on the left). Subordinates (shown on the right), downregulate the D1 receptor, leaving D3 receptors to inhibit the i-IN. This causes subsequent removal of inhibition on the M-cells and an increased in response probability.

Future experiments

The next step is to determine the location of the DA receptors within the escape circuit. Both GABA and Glycine and inhibitory neurons that are important during development of the swimming and escape circuits, and the synapses on these circuits remain through adulthood (Korn & Faber, 2005; Bailey-Cuif & Vernier, 2010; Cui et al. 2005)**.** Glycinergic receptors are primarily located on the soma of the M-cells, whereas GABAergic receptors are primarily located on the lateral dendrite (Korn & Faber, 2005). Previous research has shown the potential for DA to modulate both glycinergic and GABAergic inputs (Porras & Mora, 1993). However, DA influence on both of these inputs regarding the escape response and swim in zebrafish remains unknown. In order to determine which i-IN DA is influencing, we can first perform receptor agonist/antagonist co-injections of DA with GABA and DA with glycine to determine whether we can rescue the DA effects observed in dominants and subordinates. Another approach would be to stain for GABA and glycine located around the M-cells and then back-label those neurons to determine the location of their dendritic inputs. We can then stain for DA receptors on those dendrites. This would give us visual confirmation of the presence of these receptors that we can then conditionally target to knockout the DA receptors and determine if there are behavioral changes comparable to our pharmacological results.

Once we locate which i-IN the D1/D3 receptors are located, we can then confirm that they are forming a heterodimer by performing co-immunoprecipitation (co-IP) of D1 and D3 on this i-IN to see if they precipitate out together following the methods laid out in Fiorentini et al., (2008). In addition to co-IP, we can perform immunohistochemistry on the i-IN for a visual confirmation that both receptors appear on the same neuron. In addition, we can determine the role of the DA receptors on both the escape and swim circuits by manipulating the presence of

these receptors through a conditional CRISPR/CAS9 knockout. With this method we can target and knock out D1 and D3 receptors located only on the predetermined i-IN so that any behavioral changes we observe can be attributed to a lack of these receptors on that specific neuron. If we knock out only D1 receptors on the identified i-IN, we should see that fish display heightened escape sensitivity, resembling a subordinate phenotype. Moreover, we can confirm the presence of the i-IN connecting the escape and swim circuits, as proposed in figure 6, if we see that in addition to a heightened escape response by knocking out the D1 receptor only on the identified neuron influencing the M-cells, a decrease in swimming frequency. This result would support that when the M-cells are active, they inhibit slow MNs responsible for swimming.

Additional experiments can be performed to create a holistic understanding of how social status is modulating DA regulation of the escape and swim circuits. In this study we focused primarily on the interpretation of DA through changes in receptor activation on the escape and swim behaviors. Another avenue to be explored is how social status may be influencing presynaptic excitability of DA neurons. Here we can perform calcium imaging of DA neurons of dominants and subordinates. If we can locate the dendrites of the i-IN that DA may be modifying, we would know the location of the presynaptic DA neuron. We can use this information to determine excitability changes in those presynaptic neurons.

Overall, with this study we first showed that social status influenced how animals behave. We then determined that the status-dependent differences in zebrafish escape and swim behaviors can be regulated by the activation of dopamine receptors. More specifically, our results indicate that D1 and D3 receptors may play important roles in influencing behavioral output according to social status. Finally, our current results raise new questions that can further identify DA influence within the social status-dependent changes in escape and swimming

behaviors of zebrafish.

REFERENCES

Ali DW, Drapeau P, Legendre P (2000) Development of Spontaneous Glycinergic Currents in the Mauthner Neuron of the Zebrafish Embryo. *J Neurophysiol* 85(4):1726-1736

Ampatzis K, Song J, Ausborn J, El Manira A (2013) Pattern of innervation and recruitment of different classes of motoneurons in adult zebrafish. *J Neurosci* 33:10875-10886

Ampatzis K, Song J, Ausborn J, El Manira A (2014) Separate microcircuit modules of distinct V2a interneurons and motoneurons control the speed of locomotion. *Neuron* 83(4):934-943

Bailey-Cuif, L., Vernier, P. (2010). Organization and physiology of the zebrafish nervous system. *Fish Physiology* 29: 25-80

Brewer KL, Baran CA, Whitfield BR, Jensen AM, Clemens S. (2014). Dopamine D3 receptor dysfunction prevents anti-nociceptive effects of morphine in the spinal cord. *Front Neural Circuits* 8:62

Cabelguen JM, Bourcier-Lucas C, Dubuc R (2003) Bimodal locomotion elicited by electrical stimulation of the midbrain in the salamander Notophthalmus viridescens. *J Neurosci* 23(6):2434-2439

Canfield JG (2003) Temporal constraints on visually directed C-start responses: behavioral and physiological correlates. *Brain Behav Evol* 61:148 –158.

Chiao JY (2010). Neural basis of social status hierarchy across species. *Current Opin in Neurobio* 20(6): 803-809

Clemens S, Katz PS. (2003). G protein signaling in neuronal network is necessary for rhythmic motor pattern production. *J. Neurophysiol* 89(2):762-772

Chatterjee D & Gerlai R (2009). High precicion liquid chromatography analysis of dopaminergic and serotonergic responses to actue alcohol exposure in zebrafish. *Behav Brain Res* 200(1): 208-213

Chiara GD (1995) The role of dopamine in drug abuse viewed from the perspective of its role in motivation. *Drug and Alcohol Dependence* 38(2):95-137

Couppis MH, Kennedy CH (2008). The rewarding effect of aggression is reduced by nucleus accumbens dopamine receptor antagonism in mice. *Psychopharmacology* 197:449–456.

Cubitt KF, Winberg S, Huntingford FA, Kadri S, Crampton VO, Overli O (2008). Social hierarchies, growth and brain serotonin metabolism in Atlantic salmon (*Salmo salar*) kept under commercial rearing conditions. *Phys & Behav* 94(4): 529-535

Cui WW. Low SE, Hirata H, Saint-Amant L, Geisler R, Hume RI, Kuwada JY. (2005). The zebrafish

shocked gene encodes a glycine transporter and is essential for the function of early neural circuits in the CNS. *J. Neurosci* 25:6610-6620.

Deliagina TG, Zelenin PV, Orlovsky GN (2002) Encoding and decoding of reticulospinal commands. *Brain Res Brain Res Rev* 40(1-3):166-177

Depue RA, Collins PF (1999). Neurobiology of the structure of personality: dopamine, facilitation of incentive motivation, and extraversion

Eaton RC, Emberley DS (1991) How stimulus direction determines the trajectory of the Mauthner-initiated escape response in a teleost fish. *J Exp Biol* 161:469 – 487.

Eaton RC, Farley RD, Kimmel CB, Schabtach E (1977) Functional development in the mauthner cell system of embryos and larvae of the zebra fish. *J Neurobiol* 8(2):151-172

Eaton RC, Lee RK, Foreman MB (2001) The Mauthner cell and other identified neurons of the brainstem escape network of fish. *Prog Neurobiol* 62(4):467-485

Filby AL, Paull GC, Hickmore TF, Tyler CR (2010) Unravelling the neurophysiological basis of aggression in a fish model. *BMC Genomics* 11:498

Fiorentini C, Busi C, Gorruso E, Gotti C, Spano P, Missale C (2008) Reciprocal regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. *Mol Pharm* 74:59-69

Hamid AA, Pettibone JR, Mabrouk OS, Hetrick VL, Schmidt R, Vander Weele CM, Kennedy RT, Aragona BJ, Berke JD (2016) Mesolimbic dopamine signals the value of work. *Nature Neuro* 19:117-126

Hawkins RD (2013) Possible contributions of a novel form of synaptic plasticity in Aplysia to reward, memory, and their dysfunctions in mammalian brain. *Learn Mem* 18:20(10):580-591

Issa FA, Adamson DJ, Edwards, DH (1999) Dominants hierarchy formation in juvenile crayfish *Procambrus clarkia*. *J Exp Bio* 202:3497-3506

Issa FA, Drummond J, Cattaert D, Edwards DH (2012) Neural circuit reconfiguration by social status. *J Neurosci* 32(16):5638-5645

Izhikevich EM (2007) Dynamical systems in neuroscience: The geometry of excitability and bursting. MIT Press

Jay M, De Faveri F, McDearmid JR (2015). Firing dynamics and modulatory actions of

supraspinal dopaminergic neurons during zebrafish locomotor behavior. *Curr Bio* 25(4):435-444

Joëls M, Karst H, Harmen JK, Paul JL (2007). Chronic stress: Implication for neuronal morphology, function and neurogenesis. *Frontiers in Neuroendocrinology* 28: 72-96.

Johnsson JI (1997) Individual Recognition Affects Aggression and Dominance Relations in Rainbow Trout (Oncorhynchus mykiss). *Ethology* 104(4):267-282

Jordan BA, Devi LA. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature London* 399(6737):697-700.

Kashin SM, Feldman AG, Orlovsky GN (1974) Locomotion of fish evoked by electrical stimulation of the brain. *Brain Res* 82(1):41-47

Kvernmo T, Hartter S, Burger E (2006). A review of the receptor-binding and pharmacokinetic properties of dopamine agonists. *Clinical Therapies* 28(2):1065-1078

Kiser D, SteemerS B, Branchi I, Homberg JR (2012). The reciprocal interaction between serotonin and social behavior. *Neurosci & Biobehav Reviews* 36(2):786-798

Kohashi T, Oda Y (2008) Initiation of Mauthner- or non-Mauthner-mediated fast escape evoked by different modes of sensory input. *J Neurosci* 28(42):10641-10653

Korn H, Faber DS (2005) The Mauthner cell half a century later: a neurobiological model for decision-making? *Neuron* 47:13-28

Koyama, M., Kinkhabwala, A., Satou, C., Higashijima, S., Fetch, J. (2010). Mapping a sensory-motor network onto a structural and function ground plan in the hindbrain. *PNAS 108:1170-1175*

Lambert AM, Bonkowsky JL, Masino MA (2012). The conserved dopaminergic diencephalospinal tract mediates vertebrate locomotor development in zebrafish larvae. *J Neurosci* 32(39):13488-13500

Larson ET, O'Malley DM, Melloni RH Jr (2006) Aggression and vasotocin are associated with dominant–subordinate relationships in zebrafish. *Behav Brain Res* 167:94-102

Larson ET, Summers CH (2001). Serotonin reverses dominant social status. *Behav Brain Res* 121(1-2):95-102

Levant B (1997). The D3 Dopamine Receptor: neurobiology and potential clinical relevance. *Pharm Rev* 49(3):231-252

Li S-M, Collins GT, Paul NM, Grundt P, Newman AH, Xu M, Grandy DK, Woods JH, Katz JL (2010). Yawning and locomotor activity induced by dopamine receptor agonists in mice

and rats. *Behav Pharm* 21(3): 171-181

Maggio R, Aloisi G, Silvano E, Rossi M, Millan MJ. Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. *Parkinsonism and related disorders* 155:52-57

McLean DL, Fetcho JR (2004) Relationship of tyrosine hydroxylase and serotonin immunoreactivity to sensorimotor circuitry in larval zebrafish. *J Comp Neurol* 480(1):57-71

Medan V, Preuss T (2014) The Mauthner-cell circuit of fish as a model system for startle plasticity. *J Physiol Paris* 108(2-3):129-140

Miller TH, Clements KN, Ji G, Issa FA. (2016). Social status-dependent molecular regulation of dopaminergic pathway sin the brain of zebrafish (danio rerio). (Conference) Society for Neuro.

Miller TH, Clements KN, Sungwoo A, Choongseok P, Eoon Hye Ji, Issa FA. (2017) Social status-dependent shift in neural circuit activation affects decision making. *J. Neurosci* 37(8):2137-2148

Morris C, Lecar H (1981) Voltage oscillations in the barnacle giant muscle fiber. *J Physiol Paris* 108(2-3):129-140

Mu Q, Johnson K, Morgan PS, Grenesko EL, Molnar CE, Anderson B, Nahas Z, Kozel FA, Kose S, Knable M, Fernandes P, Nichols DE, Mailmain RB, Georse MS. A single 20 mg dose of the full D1 dopamine agonist dihydrexidine (DAR-0100) increases prefrontal perfusion in schizophrenia. *Schizophr Res* 94(1-3):332-341

Mu Y, Li XQ, Zhang B, Du JL (2012) Visual input modulates audiomotor function via hypothalamic dopaminergic neurons through a cooperative mechanism. *Neuron* 75(4):688-699

Neumeister H, Whitaker KW, Hofmann HA, Preuss T (2010) Social and ecological regulation of a decision-making circuit. *J Neurophysiol* 104(6):3180-3188

O'Connel L, Hofmann H (2012) Evolution of a vertebrate social decision making network. *Science* 336:1154-1157

Oda Y, Kawasaki K, Morita M, Korn H, Matsui H (1998) Inhibitory long-term potentiation underlies auditory conditioning of goldfish escape behavior. *Nature* 394(6689):182-185

Oliveira RF, Silva FJ, Simões JM (2011) Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. *Zebrafish* 8(2):73-81

O'Malley DM, Kao YH, Fetcho JR (1996) Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. *Neuron* 17(6):1145-1155

Paull GC, Filby AL, Giddins HG, Coe TS, Hamilton PB, Tyler CR (2010) Dominance hierarchies in zebrafish (Danio rerio) and their relationship with reproductive success. *Zebrafish* 7:109-117

Pavlidis M, Sundvik M, Chen YC, Panula P (2011) Adaptive changes in zebrafish brain in dominant-subordinate behavioral context. *Behav Brain Res* 225(2):529-537

Pavlik LL, Bezgina EN, Tiras NR, Mikheeva IB, Udal'tsov SN, Moshkov DA (2005) The Structure of Mixed Synapses in Mauthner Neurons During Exposure to Substances Altering Gap Junction Conductivity. *Neurosci Behav Physiol* 35(5):447-452

Peng J, Zhang J, Meng A. (2012) The tiny zebrafish keep swimming fast in the developmental pond. *J Genet Genomics* 20:39(9)419-420

Pereda A, Triller A, Korn H, Faber D (2005) Dopamine enhances both electrotonic coupling and chemical excitatory postsynaptic potentials at mixed synapses. *Proc Natl Acad Sci USA* 89(24):12088-12092

Pereda A, Triller A, Korn H, Faber DS (1992) Dopamine enhances both electrotonic coupling and chemical excitatory postsynaptic potentials at mixed synapses. *Proc Natl Acad Sci USA* 89(24):12088-12092

Pereda AE, Nairn AC, Wolszon LR, Faber DS (1994) Postsynaptic Modulation of Synaptic Efficacy at Mixed Synapses on the Mauthner Cell. *J Neurosci* 14(6):3704-3712

Platt ML, Seyfarth RM, Cheney DL (2016) Adaptations for social cognition in the primate brain. *Philos Trans R Soc Lond B Biol Sci* 371(1687):20150096

Porras A, Mora F. (1993). Dopamine antagonist blocks the release of glycine, GABA, and taurine produced by amphetamine. *Brain Res Bull* 31(1-4):305-310

Preuss T, Osei-Bonsu PE, Weiss SA, Wang C, Faber DS (2006) Neural representation of object approach in a decision-making motor circuit. *J Neurosci* 26:3454 –3464

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia A-S, White LE (2012). *Neuroscience* (5th ed.). Sunderland, MA:Sinauer Associates, INC

Ryczko D, Gratsch S, Auclair F, Dube C, Bergeron S, Alpert MH, Cone JJ, Roitman MF, Alford S, Dubuc R (2013). Forebrain dopamine neurons project down to a brainstem region controlling locomotion. *PNAS* doi:10.1073/pnas.1301125110

Sapolsky, R.M. (2004). Social status and health in humans and other animals. *Annual Review*

Anthropology 33: 393-418

Severi KE, Portugues R, Marques JC, O'Malley DM, Orger MB, Engert F (2014) Neural control and modulation of swimming speed in the larval zebrafish. *Neuron* 83(3):692-707

Sidhu A, Niznik HB. Coupling of dopamine receptor subtypes to multiple and diverse F proteins. *In J Dev Neurosci* 18:669-677

So N, Franks B, Lim S, Curley JP (2015) A Social Network Approach Reveals Associations between Mouse Social Dominance and Brain Gene Expression. *PLoS ONE* 10(7): e0134509. https://doi.org/10.1371/journal.pone.0134509

Song J, Ampatzis K, Ausborn J, El Manira A (2015) A hardwired circuit supplemented with endocannabinoids encodes behavioral choice in zebrafish. *Curr Bio* 25:2610-2620

Tan EK, Jankovic J (2001). Choosing dopamine agonists in Parkinson's disease. *Clin Neuropharmacol* 24:247-253

Thiele TR, Donovan JC, Baier H (2014) Descending control of swim posture by a midbrain nucleus in zebrafish. *Neuron* 83(3):679-691

Teles, M.C., Dahlbom, S.J., Winberg, S.,Oliveira, R.F. (2013) Social modulation of brain monamine levels in zebrafish. *Behavioral Brain Research 253:*17-24

Venables WN, Ripley BD (2002) Modern Applied Statistics with S. Fourth Edition. Springer, New York. ISBN 0-387-95457-0:

Wang WC, McLean DL (2014) Selective responses to tonic descending commands by temporal summation in a spinal motor pool. *Neuron* 83(3):708-721

Watanabe N, Yamamoto M (2015) Neural mechanisms of social dominance. *Front Neurosci* 9:154. https://doi.org/10.3389/fnins.2015.00154

Whitaker KW, Neumeister H, Huffman LS, Kidd CE, Preuss T, Hofmann HA (2011) Serotonergic modulation of startle-escape plasticity in an African cichlid fish: a single-cell molecular and physiological analysis of a vital neural circuit. *J Neurophysiol* 106(1):127-137

Yamaguchi Y, Lee YA, Kato A, Goto Y (2016) The Roles of Dopamine D1 Receptor on the Social Hierarchy of Rodents and Nonhuman Primates. *Int J Neuropsychopharmacol* pyw106:

Yamaguchi Y, Lee YA, Kato A, Jas E, Goto Y (2017) The roles of Dopamine D2 Receptor on Social Hierarchy of Rodents and Nonhuman Primates. *Scientific Reports* 7(43348):1-10. Doi: 10.1038/srep43348

Yamamoto K, Vernier P (2011) The evolution of dopamine systems in chordates. *Front Neuroanat* 5:21

Yao C, Vanderpool KG, Delfiner M, Eddy V, Lucaci AG, Soto-Riveros C, Yasumura T, Rash JE, Pereda AE (2014) Electrical synaptic transmission in developing zebrafish: properties and molecular composition of gap junctions at a central auditory synapse. *J Neurophysiol* 112(9):2102-2113

Yeh SR, Fricke RA, Edwards DH (1996) The effect of social experience on serotonergic modulation of the escape circuit of crayfish. *Science* 271:366 –369

Zhang J, Xiong B, Zhen X, Zhang A (2008). Dopamine D1 receptor ligands: where are we now and where are we going. *Medicinal Res Rev* 29(2):272-294

Zeng C, Wang D, Yang Z, Wang Z, Asico LD, Wilcox CS, Eisner FM, Welch WJ, Felder RA, Jose PA. (2004). Dopamine D1 receptor augmentation of D3 receptor action in rat aortic or mesenteric vascular smooth muscles. *Hypertension* 43:673-679

Zottoli SJ (1977) Correlation of the startle reflex and Mauthner cell auditory responses in unrestrained goldfish. *J Exp Biol* 66:243-254

Zottoli SJ, Faber DS (2000) The Mauthner cell: what has it taught us? *Neuroscientist* 6:26-38

Zottoli SJ, Marek LE, Agostini MA, Strittmatter SL (1987) Morphological and physiological survival of goldfish Mauthner axons isolated from their somata by spinal-cord crush. *J Comp Neurol* 255:272–2

APPENDIX: IACUC APPROVAL LETTERS

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 1, 2014

Fadi Issa, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Issa:

Your Animal Use Protocol entitled, "Developmental and Functional Effects of Spinocerebellar Ataxia Type-13 on Zebrafish Cerebellum and the Effects of Social Experience on Zebrafish Startle Escape Response" (AUP #D320) was reviewed by this institution's Animal Care and Use Committee on 12/1/14. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

MEMORANDUM

TO: Katie Clements
Department of Biology

FROM: Dorcas O'Rourke, D.V.M.
University Veterinarian

SUBJECT: Certificate of Training
Training Date 10/8/14

DATE: October 8, 2014

This letter is provided to certify that you have completed training in humane methods of animal experimentation, proper handling of selected species of research animals, and methods for reporting deficiencies in animal care and treatment. The training was provided in accordance with U.S. Department of Agriculture (9 CFR 2.32) regulations and the Public Health Service Policy.

This training included information on ECU animal care organizational structure, regulatory requirements, IACUC procedures, program for veterinary and animal care, occupational health and safety program, and methods for reporting concerns. Information on biology and care, proper restraint and procedures, and allergies and zoonoses were also provided.

We suggest that you retain this letter in your training file for future reference.