SOCIAL REGULATION OF THE ENDOCANNABINOID SYSTEM IN ZEBRAFISH MOTOR CIRCUITS

By

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Social status-dependent modulation of neural circuits has been investigated extensively in vertebrate and invertebrate systems. However, the effect of social status on shifting the balance in activation between competing neural circuits is poorly understood. Zebrafish (*Danio rerio*) form stable social relationships that consist of socially dominant and subordinate animals. Once the social hierarchy is formed, social status-dependent differences in behavior patterns emerge. Subordinate animals startle more readily in response to auditory stimuli, while dominants swim at a higher frequency than subordinates. Here, we investigated the role of the endocannabinoid system (ECS) in regulating the activation of the swim and escape circuits based on social status. Our aim was to investigate how the ECS facilitates the transition between swim and escape circuits in socially dominant and subordinate animals. Endocannabinoids act as retrograde signaling molecules between neurons and are implicated in inhibition of both excitatory and inhibitory neurotransmission via retrograde binding of the cannabinoid 1 (CB1) or cannabinoid 2 (CB2) receptor. A previous study revealed a novel role for the endocannabinoid 2-Arachidonoylglycerol (2-AG) in modulating the switch in activation between the swim and startle circuits in zebrafish. The ECS can be up- or down-regulated by altering levels of 2-AG or targeting CB1 receptor function. To better understand how social status regulates the ECS and its effects on circuit

activation, we studied the effects of two drugs, AM-251 and JZL184, on the regulation of status-dependent differences in swim and escape behavior. AM-251 competitively blocks endocannabinoid signaling by binding to CB1 receptor, while JZL184 increases 2-AG concentration by inhibiting monoacylglycerol lipase (MAGL), the degradative enzyme for 2-AG. First, we show that increasing ECS activity via intramuscular injection of JZL184 differentially affects swim and escape behavior according to social status. Secondly, we show that block of CB1 function with AM-251 reduces startle sensitivity and swimming frequency, and that its effects are concentration dependent. Thirdly, we utilize a dopamine receptor 1 knockout fish (D1KO) to demonstrate that the effects of ECS modulation on startle involves the dopamine D1 receptor system. Collectively, these findings support the notion that the ECS, as reflected by changes in swimming and escape behavior in response to treatment with JZL184 and AM-251, is socially regulated and involved in the social status-dependent shift in the balance of motor circuit activation, and that these effects are mediated in part via dopaminergic pathways. Our results represent an important step forward in the field of social neuroscience and better define the path toward a comprehensive understanding of the molecular factors that control social behavior.

SOCIAL REGULATION OF THE ENDOCANNABINOID SYSTEM IN ZEBRAFISH MOTOR CIRCUITS

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This work is dedicated to my mother, whose encouragement was vital to my pursuit of a Master's degree, all the members of the Issa lab, and my thesis committee members for their guidance.

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LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
CB1	Cannabinoid 1 Receptor
CB2	Cannabinoid 2 Receptor
CPG	Central pattern generator
D1	Dopamine 1 Receptor
DA	Dopamine
DAG	Diacylglycerol
DAGL	Diacylglycerol Lipase
ECS	Endocannabinoid System
EPSP	Excitatory Post-synaptic Potential
GPCR	G-protein coupled receptor
i-IN	inhibitory interneuron
e-IN	excitatory interneuron
M-Cell	Mauthner Command Neuron
MAGL	Monoacylglycerol lipase
mGluR1	Metabotropic glutamate receptor (group 1)
MN	Motor neuron
PKA	Protein kinase A
PLC	Phospholipase C
PTN	Posterior tubercular nucleus
VIIIth Nerve	Vestibulocochlear sensory nerve

GENERAL INTRODUCTION

Animal social behavior is dependent on genetic, physiological and environmental factors. (O'Connel and Hofmann, 2012). Physiological conditions are influenced by external stimuli – both social and asocial – leading to behavioral responses that ideally provide survival benefits. Many social species, both invertebrates and vertebrates, organize themselves hierarchically by means of aggressive encounters (Edwards and Kravitz 1997; Issa et al., 1999). This system, in which some animals are subordinate to others, provides social stability, and its natural consequence is an unequal distribution of resources and mating rights among individuals in a population (Sapolsky, 2005). The hierarchical social organization of individuals in a population is made possible by inter-individual transmission of social information. This transfer of information requires a nervous system with sufficient plasticity to respond appropriately to a dynamic social environment.

Social status can be defined by the set of behaviors that accompanies a particular position in the social hierarchy. Aggressive behavior typically displayed by dominant animals consist of either physical attacks or pursuit of other members of the population. The animal that attacks more frequently will assume a dominant relationship to subordinates that retreat. This general phenomenon occurs in both invertebrates (Issa et al. 1999) and vertebrates, including zebrafish (Miller et al. 2017). Over extended periods of time, aggressive encounters differentially affect motor behavior. Socially subordinate animals display characteristic changes in behavior to signal their subordination and mitigate attacks from dominant individuals (Neumeister et al. 2010; Bosch-Bouju 2016; Miller et al. 2017).

Zebrafish as a model organism for studying social behavior. Zebrafish (*Danio rerio*) has emerged as a useful model system for a wide range of biomedical studies. Notable advantages of zebrafish include their rapid development to adulthood and easy maintenance. The external development and translucence of the embryos makes zebrafish an ideal system to study the development of internal structures and allows for easy genetic manipulation (Oliveira et al., 2011). Another quality of zebrafish that makes them useful in behavioral studies is that they are highly social animals. When two adult male zebrafish are paired in a tank, they quickly establish a stable social relationship in which one fish is dominant and the other subordinate. These social relationships can be used as the basis to study the effects of social status on behavior and brain function (Miller et al. 2017).

Two fundamental behaviors in zebrafish - startle and swimming - are notable for the relative simplicity of the neural circuits that control these behaviors and the ease with which they can be studied behaviorally and physiologically. The neural circuits underlying these basic motor behaviors have been well-characterized in terms of their neuronal organization (Eaton et al. 2001) and the neurochemicals that modulate their activation (McLean and Fetcho 2004). The startle response in zebrafish and other teleost fish is controlled by a group of reticulospinal neurons, namely the Mauthner cell (M-cell) and two serial homologs, MiD2cm and MiD3cm. The primary components are the two contralateral M-cells – one on either side of the brain. The firing of a single M-cell is necessary and sufficient for the initiation of a fast startle response. The M-cells act as integration centers for auditory, tactile, and visual inputs, and, as such, they are responsible for the initiation of startle behavior in response to auditory stimuli (Eaton et al. 2001). Auditory stimuli activate hair cells in the ear, which then send a signal along the VIIIth cranial nerve to the M-cell. A stimulus sufficient to activate the M-cell subsequently activates fast motor neurons

(MNs) and inactivates slow MNs, generating a nervous system-induced contralateral contraction of the trunk musculature (Eaton et al. 2001). This produces a fast escape (C-start) away from the stimulus (Figure 1A).

Swimming is a well-conserved behavior that has the same basic neural circuit in many aquatic vertebrates. The swimming circuit has been described in lamprey (Grillner et al. 1996), frog tadpoles (Roberts et al. 2008), and zebrafish (Kiehn 2011; Fetcho and McLean 2010). It is controlled by a distributed network of neurons arranged hierarchically from the midbrain to the spinal cord. Initiation of locomotion begins in the mesencephalic locomotor region (MLR). The MLR sends descending inputs to reticulospinal neurons in the hindbrain, which project to the central pattern generators (CPGs). The CPG consists of two half-centers, one on either side of the midline (Figure 1B). Each half-center is composed of motor neurons, descending excitatory interneurons (e-INs), and commissural inhibitory interneurons (i-INs). The coordinated action of these neurons is responsible for the locomotor pattern generation (Roberts et al. 2008).

The balance between the swimming and startle circuits in zebrafish is known to be regulated by social status (Miller et al. 2017). However, the complete array of neurochemical changes that mediate the effects of social factors on behavior remain incompletely understood. Serotonin has been the focus of studies on social behavior due to its known connection to aggression (Larson and Summers, 2001; Chiao et al. 2010; Kiser et al., 2012). We are less concerned with the neural systems that control the expression of aggression, and more concerned with motor systems that respond to aggression by inducing alterations in behavior. More specifically, we are interested in chemical systems that facilitate the shift in balance between startle and swimming based on social status. The neuromodulator dopamine has been implicated in the formation of social hierarchies (Watanabe and Yamamoto 2015) and targeting the dopaminergic

system has been shown to affect swimming and startle in a social status-dependent manner (Clements 2017). While the roles of the serotonergic and dopaminergic systems in determining social behavior are well-described, the role played by the endocannabinoid system (ECS) has yet to be explored. We decided to focus our attention on the ECS, which is directly involved in switching activation between competing neural circuits (Song et al. 2015).

The switching between the startle and swim circuits is controlled by a hardwired neural circuit spanning from the hindbrain to the spinal cord. However, the threshold for the switch from swimming to startle was recently discovered to be modulated by endocannabinoids (Song et al. 2015). The retrograde system of neurochemicals known as the ECS plays an integral part in balancing the activation between motor circuits in zebrafish. Using a combination of electrophysiology and pharmacology, Song and colleagues showed that the endocannabinoid 2-arachidonoylglycerol (2-AG) sets the threshold for the switch from swimming to startle. Another prominent study in goldfish demonstrated that the reticulospinal M-cells release 2-AG in order to regulate their own excitability (Cachope et al., 2007). However, nothing is known about what role, if any, endocannabinoids play in social status-dependent changes reflected in two distinct motor behaviors. Here, we investigated how social status affects regulation of neural circuit activation of the startle and escape behaviors mediated by the ECS. Given that the ECS was shown to modulate the transition between escape and swim (Song et al. 2015), and that social status shifts the activation pattern of the escape and swim responses (Miller et al. 2017), we hypothesize that the ECS is involved in regulating social status-dependent behavior.

Thesis Outline. The primary objective of this study was to understand whether the ECS is involved in the shift in motor circuit activation induced by social status. We first determined the role of the ECS in regulating the social status-induced changes in the neural circuits underlying startle and swimming behaviors using a pharmacological approach. We found strong evidence that the ECS is integral to the expression of different behavior patterns characteristic of dominant and subordinate animals. We also record that the behavioral effects of drugs targeting the ECS depend on the concentration of the drug.

Next we present evidence supporting the involvement of the dopaminergic system in the regulatory effects of the ECS on swimming and startle behavior. We use a dopamine receptor knockout zebrafish line to demonstrate that the effects of ECS modulation of motor behaviors is dependent on intact dopaminergic signaling.

The discussion synthesizes our results to provide a hypothetical model of how the ECS and dopaminergic system interact to regulate status-dependent behavior. We review the relevant literature to explore connections between the ECS and socially determined behavior. We discuss how differential ECS activity in the swimming circuit of dominant and subordinate fish underlies differences in swimming frequency. We further discuss our results showing different effects of ECS manipulation on the startle behavior of different social types. We then propose a model to explain how the ECS and dopaminergic system may induce status-dependent escape behavior by altering the molecular makeup of M-cell inputs. Finally, we propose several future experiments that would further elucidate the interactions between the ECS and dopaminergic systems, and also explore the role of the ECS in the expression of aggressive behavior.

GENERAL METHODS

Animal maintenance. Zebrafish (*Danio* rerio) were housed at the Zebrafish Core Facility at East Carolina University. The facility was kept at a constant temperature of at 28°C under a 14 h/10 h light/dark cycle. Fish were fed twice daily with a high protein commercial food (Otohime B2, Reed Mariculture, CA, USA), and once daily with newly hatched artemia (Brine Shrimp Direct, UT, USA). Fish were group-housed in 10 gallon mixed-sex tanks prior to isolation and pairing. All experiments were performed in accordance with the Institutional Animal Care and Use Committee at East Carolina University (AUP #D320).

Social isolation and pairing. Male fish were taken from their communal tanks and isolated in a tank for 1 week, separated spatially and visually from other fish. This protocol was shown to minimize pre-existing social status (Miller et al. 2017). After this, social isolates of equal size and age were paired in a new tank over a 2-week period and behavior monitored as described in Experimental setup.

Experimental setup. After the pairing phase was complete fish were temporarily separated, and behavioral testing was performed on a single fish following the protocol described by Issa et al. (2011). Each fish was placed in a testing chamber (dimensions: 11 x 4 x 3cm) filled with double distilled water having a resistance of ~15 MΩ • cm (Figure 2A). High resistance water allows for more sensitive detection of field potentials and prevents dissipation of the electrical signals. It has no adverse effects on the health of the fish (Issa et al., 2011; Monesson-Olson et al., 2014). A pair of conductive electrodes placed on either side of the chamber recorded the electric field potentials. Bare electrodes were 1 mm in thickness with 3-5 mm metal exposure. Electrodes were connected to an AC differential amplifier (AM-Sytems model 1700, Carlsborg, WA USA), allowing the

amplification of signals 1000-fold. Electrical signals were low-pass filtered at 300 Hz and high-pass filtered at 1 KHz. Electrical field potentials are generated by muscle contractions when the fish moves (Issa et al. 2011). These signals were digitized using a Digidata-1322A digitizer then stored using Axoscope software (Molecular Devices, Inc., Sunnyvale, CA, USA). The experimental animals were acclimatized for 30 minutes before behavioral testing was initiated. Swimming behavior was recorded immediately following acclimation. Immediately after, startle responses were recorded.

Determination of startle sensitivity. Auditory pulses consisting of phasic 4 ms sine waves were generated using Audacity open source audio editor and recorder software (audacityteam.org). Sound intensity was measured and calibrated external to the tank using a decibel meter (Sinometer, MS6700). Sensitivity of the animal's auditory startle response was determined by tracking startle probability as a function of sound intensity. Activation of the Mauthner-mediated escape is an all or nothing response with a short latency from stimulus (5-15 ms), and startles were only recorded if they fell within this range (Figure 2B). Non-Mauthner mediated responses with a time onset ranging from 15-40 ms were not counted, as these are controlled by an independent set of neural circuit that is not the target of our investigation (Eaton et al. 2001). Pulse intensity ranged from 70-100 dB with 5 dB increments. Pulses were randomly presented according to intensity with a minimum of 2-minute intervals between trials to prevent habituation of the startle reflex. Pulses had the following distribution: 70 dB x 1; 75 dB x 3; 80 dB x 5; 85 dB x 4; 90 dB x 3; 95 dB x 1; 100 dB x 1. Response probability for each intensity was tabulated, and these probabilities were averaged across animals.

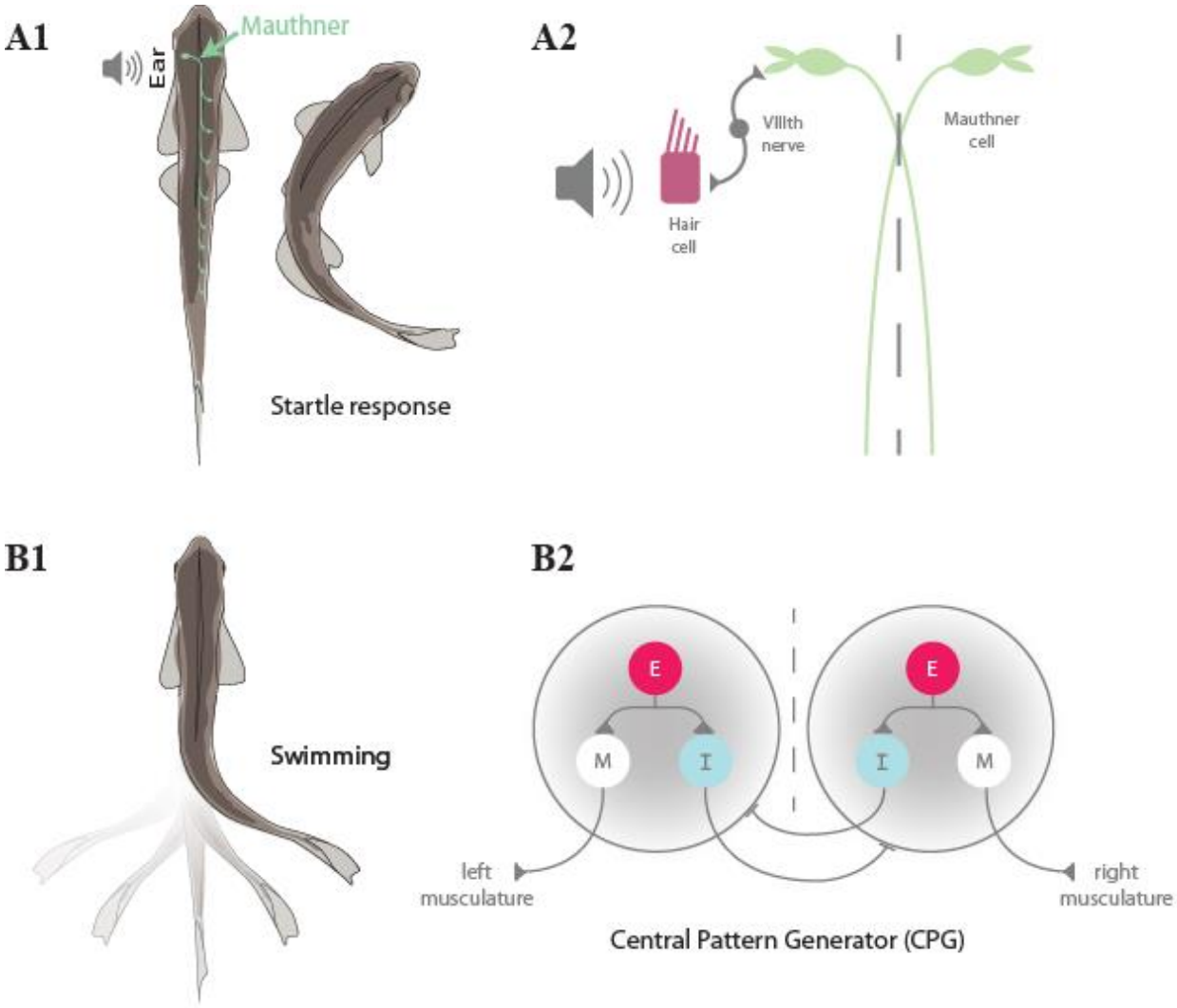
Measurement of swimming activity. Following the 30-minute acclimation period, and before conducting startle experiments, the animal's swimming behavior was recorded for 1 minute. The

same methods of data acquisition, amplification, digitization, and storage were used as previously stated. Swimming activity was measured by counting swim bursts with Clampfit software. The "Threshold" function was used for this purpose. A potential was marked as a swim burst if it was at least 8 mV in total amplitude and 30-200 ms in duration. This range was chosen based on the typical characteristics of rhythmic swimming potentials that we observed. The timing of each swim burst was saved into a Microsoft Excel spreadsheet in reference to the recording start time.

Data Analysis. Startle and swimming behavioral data was analyzed using Prism (GraphPad software Inc., San Diego, USA). All comparisons were first subjected to one-way analysis of variance testing (ANOVA) or mixed design (a mixture of one between-group and repeated measures variables) ANOVA followed by least significant difference (LSD) post hoc test for all multiple comparisons. For startle data, nonlinear regressions were performed using the Boltzmann sigmoidal equation: $Y = Bottom + (Top - Bottom)/(1 + exp((V50 - X)/Slope))$.

Pharmacology. A day after initial behavioral testing, fish were treated with either AM-251 or JZL184 and re-tested according to the previously stated protocol. Paired fish were separated with a divider during the injection and post-testing phase. The acclimation period was initiated 2 hours post-injection. Fish were treated with a drug injected intraperitoneally following the protocol of Song et al. 2015. Intraperitoneal injections are preferred over direct brain injections (1) because there is less risk of altering behavior with the physical injection and (2) because both drugs can effectively cross the blood-brain barrier (Song et al. 2015). The drugs AM-251 and JZL184 were dissolved in DMSO to produce a 40 mM stock solution. For injection, capillary tubing was used, having the dimensions 1.0 mm OD x 0.5 mm ID x 100 mm in length. These were pulled using Flaming/Brown Micropipette Puller – Model P-87 from Sutter Instrument Co. The 40 mM stock solution was diluted in saline to 400 μM AM-251 and 400 μM JZL184. The tip of the micropipette

was broken off with a razor blade, before loading with the drug solution. Loaded micropipettes were placed in Pneumatic PicoPump PV 820 for drug administration. A 0.3 % tricaine solution was used to anaesthetize the animal prior to injection. Zebrafish were determined to have an average weight of 100mg, therefore 2 μL of drug was injected to achieve a concentration of 4mg/kg AM-251 and 4mg/kg JZL184. To control for injury from injection and possible effects from solvents, separate dominant-subordinate pairs were injected with 10% DMSO in saline. To control for social status, communal fish were injected with either AM-251 or JZL184.



Designed by Kristen Orr

Figure 1 - Zebrafish escape and swim circuits

A) Startle behavior in zebrafish is controlled by the Mauthner startle circuit. The auditory startle response is activated when a sound activates hair cells within the ear. Next, the signal is sent from the VIIIth nerve to the Mauthner cell, which activates contralateral fast motor neurons responsible for contraction of flexor muscles that leads to the startle response. B) The swimming motor pattern is controlled by the central pattern generators (CPGs) which repeat along the length of the spinal cord. Each half-center of the CPG is composed of an excitatory interneuron (E), an inhibitory interneuron (I), and a motor neuron (M). The motor neurons project ipsi-laterally to the trunk musculature and induce contraction.

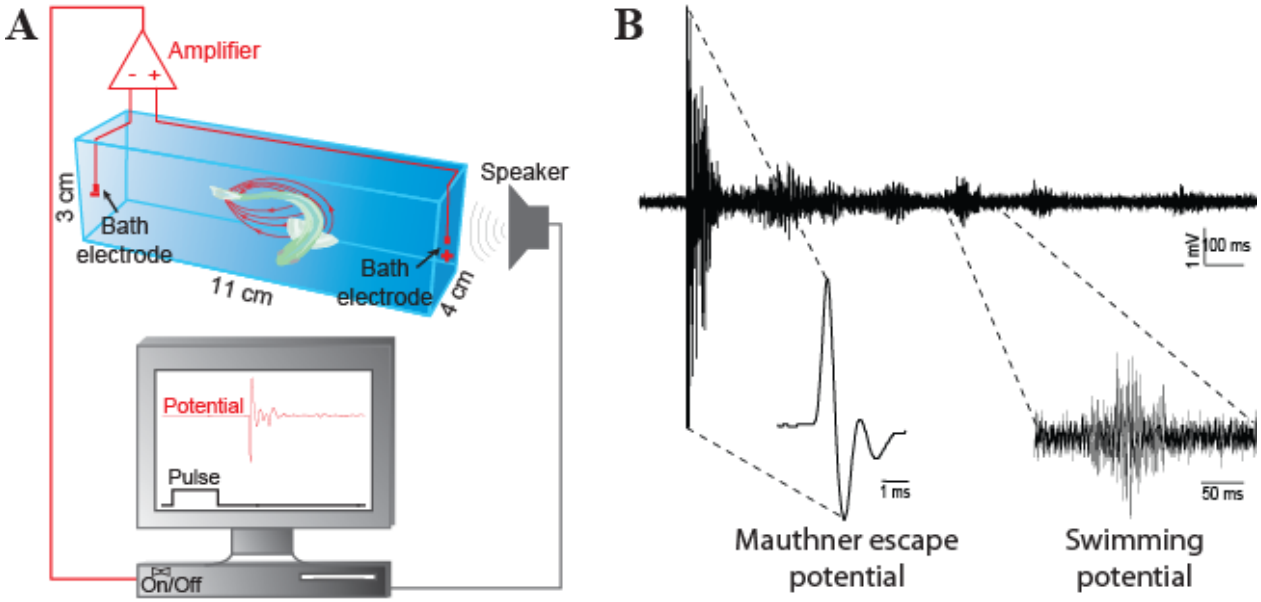


Figure 2 - Far-field potential recording of escape and swim responses in freely behaving animals

A) A fish is placed in a small container filled with reverse osmosis water. Bath electrodes placed on opposite sides of the container pick up field potentials produced when the fish moves. The high-resistance water prevents dissipation of the field potentials. These potentials are amplified and digitally recorded. B) Example traces of field potentials. The first type is the high amplitude, short duration Mauthner escape potential, produced when the fish startles; the second is the low amplitude, long duration swim burst produced during rhythmic swimming.

CHAPTER ONE: INTRODUCTION

In this study, we investigated the effects of ECS modulation on the social-status dependent activation of two competing motor circuits: escape and swim circuits. The ECS is remarkable both for its unique retrograde signaling mechanism (Figure 3) and for the scope of its involvement in nervous system function. The ECS is composed of cannabinoid receptors and their endogenous lipid-based ligands, i.e., endocannabinoids. Two cannabinoid receptors have been identified in vertebrates, the cannabinoid 1 receptor (CB1) and the cannabinoid 2 receptor (CB2). While CB1 is the primary cannabinoid receptor found in the brain, CB2 is also present, although at much lower receptor number per cell and is found primarily on immune cells (Lam et al. 2006). The endogenous ligands anandamide and 2-AG are retrograde signaling molecules, which are synthesized "on demand" in response to post-synaptic depolarization (Kano et al., 2009). The synthesis of 2-AG in the post-synaptic neuron is triggered by intracellular increase in Ca^{2+} concentration resulting from cell depolarization. Binding and activation of presynaptic CB1 leads to the pre-synaptic closing of Ca^{2+} channels and/or opening of K^{+} channels. These cellular changes result in reduced neurotransmitter release (Hernandez and Cheer 2015). After being transported into the presynaptic neuron by an unknown uptake mechanism (Fu et al., 2011), 2-AG is degraded by monoacylglycerol lipase (MAGL) as a mechanism to regulate 2-AG activity (Dinh et al., 2002). The ECS is involved in a wide range of brain functions, including memory (Lupica et al., 2017), motivation (Covey et al., 2017), and sensation (Woodhams et al. 2017). Moreover, CB1 receptors have even been found on mitochondrial membranes, suggesting a role in the regulation of energy metabolism (Araque et al. 2017). However, our particular focus is on how social experience regulates the ECS activation of motor circuits.

A recent study focusing on the spinal cord circuit in zebrafish demonstrated that the endocannabinoid 2-arachidonoyl glycerol (2-AG) acts as a molecular "clutch" that sets the threshold for the switch from swimming to startle behavior (Song et al. 2015). Furthermore, evidence strongly suggests that the M-cell releases 2-AG (Cachope et al. 2007). It was found that activation of the group 1 metabotropic glutamate receptor (mGluR1) led to a lasting potentiation from the VIIIth nerve onto the M-cell. 2-AG is known to be synthesized and released from a post-synaptic cell in response to mGluR1 activation. Moreover, it was found that blocking CB1 eliminated this potentiation. They concluded that the M-cell increases its own excitability by releasing 2-AG. The findings from these two studies set the stage to study the role of the ECS in balancing activation of the startle and swimming circuits based on social status.

Switching between mutually exclusive behaviors is a fundamental biological process that enables behavioral adaptation to a changing environment. A thorough knowledge of behavioral switching - otherwise known as decision-making - would have wide-ranging applications. To understand how higher vertebrates make decisions, or switch between two mutually exclusive behaviors, we must first understand the neural underpinnings of switching activation between competing circuits in a simple neural system.

When considering decision-making in social animals, it is impossible to account for the full repertoire of behavior by considering an animal in isolation. The balance between competing behaviors can only be fully understood in the context of the animal's social environment. Social status-dependent modulation of neural circuits has been investigated extensively in vertebrate and invertebrate systems (Edwards and Kravitz 1997; Whitaker et al. 2011). External social factors can shift the balance in favor of one behaviorally relevant output over another by modulating their respective circuits. When paired, adult male zebrafish engage in aggressive behavior that results

in a stable social relationship (Miller et al. 2017). Socially dominant fish had lower startle sensitivity and higher swimming frequency, whereas socially subordinate fish showed a shift in circuit activation towards higher sensitivity of the Mauthner startle reflex and lower activation of the swimming circuit resulting in lower swimming frequency.

While the effects of social status on behavior are well-documented, the effects of social status on the molecular machinery responsible for shifting activation between the two competing neural circuits of escape and swim is poorly understood. The known role of the ECS in switching activation between motor circuits suggests that it could be involved in the facilitation of social status-dependent shifts in behavior. We wanted to know whether ECS modulation of motor circuit activation depended on social status. There were three major aims of this study. First, we set out to replicate experiments of Song et al. (2015), which implicated the ECS in the process of shifting the balance in activation between the two competing motor circuits responsible for startle and swimming. Second, we aimed to expand these findings to encompass social status-dependent shifts in their activation threshold. And finally, we sought to determine whether ECS regulation of the activation pattern of motor behavior is mediated by dopaminergic signaling.

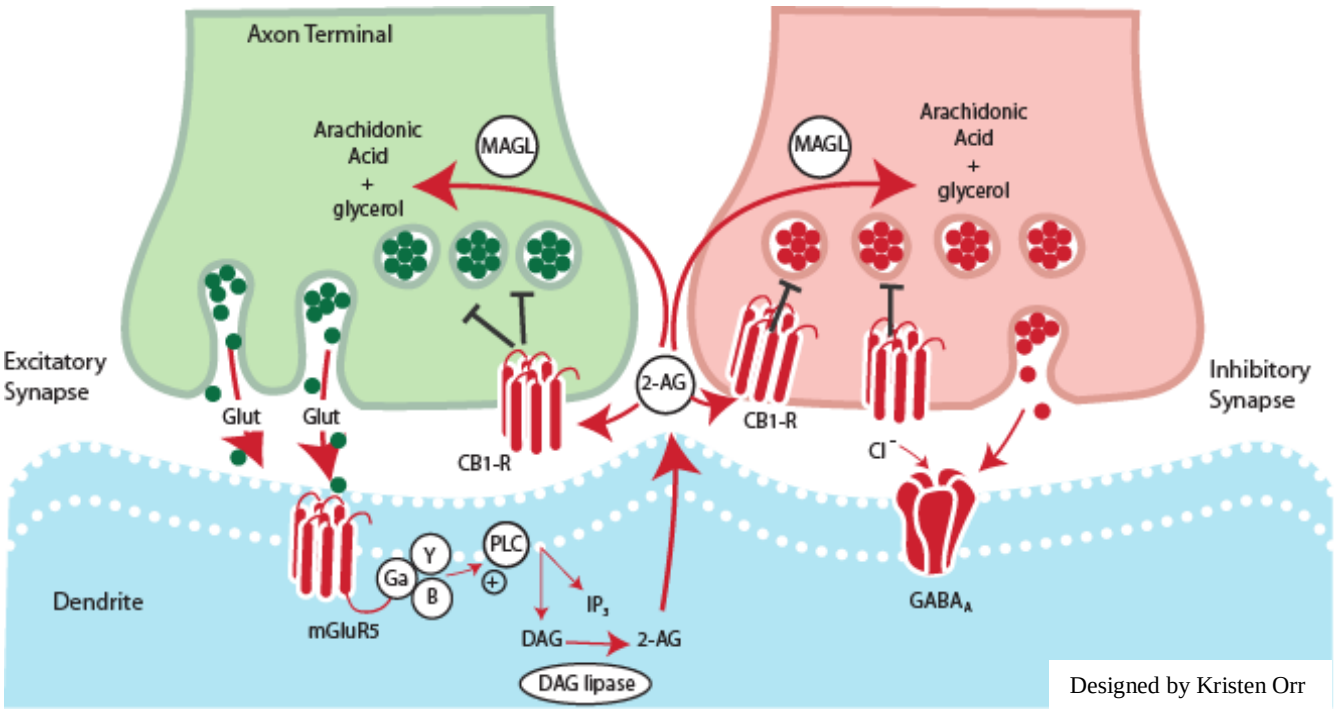


Figure 3 – Endocannabinoid system (ECS) overview

General schematic model of canonical endocannabinoid retrograde signaling. The endocannabinoid 2-AG is synthesized in the post-synaptic cell dendrite (blue) in response to neurotransmitter binding. 2-AG travels back across the synapse to inhibit further release from both excitatory terminals (green) and inhibitory terminals (red). DAG lipase synthesizes 2-AG. CB1 receptor binds 2-AG. MAGL degrades 2-AG in presynaptic terminal.

CHAPTER TWO: RESULTS

Social status regulation of startle and swimming. To verify previous findings by Miller and colleagues (2017), we replicated their fundamental discovery that social status alters motor behavior in zebrafish. We found that, indeed, subordinate fish have a lower startle threshold than dominants or communal fish (Figure 4A-B). Using a Mann Whitney test, subordinates were found to have a lower startle threshold than dominants at 75dB ($p=0.0391$), 80dB ($p=0.0011$), 85dB ($p=0.0001$), and 90dB ($p=0.0361$). The Mann Whitney test also revealed that subordinates have a lower startle threshold than communals at 80dB ($p=0.0034$) and 85dB ($p=0.0002$). Next, it was found that dominants swim at a higher frequency than subordinates (Mann Whitney test, $p=0.0002$) and communals (Mann Whitney test, $p=0.0179$; Figure 4C-D). There was no significant difference in swimming rate between subordinates and communals. Based on these robust results showing that social status influences startle and swimming, we hypothesized that the regulatory effects of endocannabinoids on motor behavior depend on social status.

Status-dependent influences of ECS modulation on startle sensitivity. To determine whether ECS modulation of the startle escape circuit is socially regulated, we pharmacologically manipulated 2-AG systemic availability by either injecting JZL184 or AM-251 (Figure 5). In communal fish, inhibiting the breakdown of 2-AG through JZL184 significantly increased startle sensitivity at 85dB but not at 75 or 80 dB (Wilcoxon match pairs t-test, $p=0.0156$; Figure 5A). JZL184 yielded opposite effects on startle behavior between dominants and subordinates. Dominants showed a significant enhancement in startle sensitivity at 85dB only (Wilcoxon match pairs t-test, $p=0.0391$; Figure 5B). In contrast, subordinates showed a marked reduction in startle sensitivity at 80dB and 85dB but not at 75 dB (Wilcoxon match pairs t-test, $p=0.0029$ at 80dB,

p=0.0313 at 85dB; Figure 5C). Post-injection startle sensitivity between dominant and subordinate fish was not significantly different. These results point to the central role of 2-AG in mediating expression of social status-dependent differences in startle sensitivity.

Suppressing ECS activity with injection of the CB1 receptor antagonist AM-251 (400μM) did not result in social status-dependent effects. Blocking CB1 in either communal or dominant fish induced a non-significant trend of reduced startle behavior (Figure 5D, E). Blocking CB1 in subordinate fish similarly reduced startle, but with a significant reduction in sensitivity at 80dB (Wilcoxon match pairs t-test, p=0.0078; Figure 5F). This result suggests one of two things: 1) there is no social status-dependent difference in CB1 receptor concentration on the inputs to the M-cell, or 2) the high concentration of AM-251 saturated the receptors in both dominants and subordinates regardless of differences in CB1 receptor concentration.

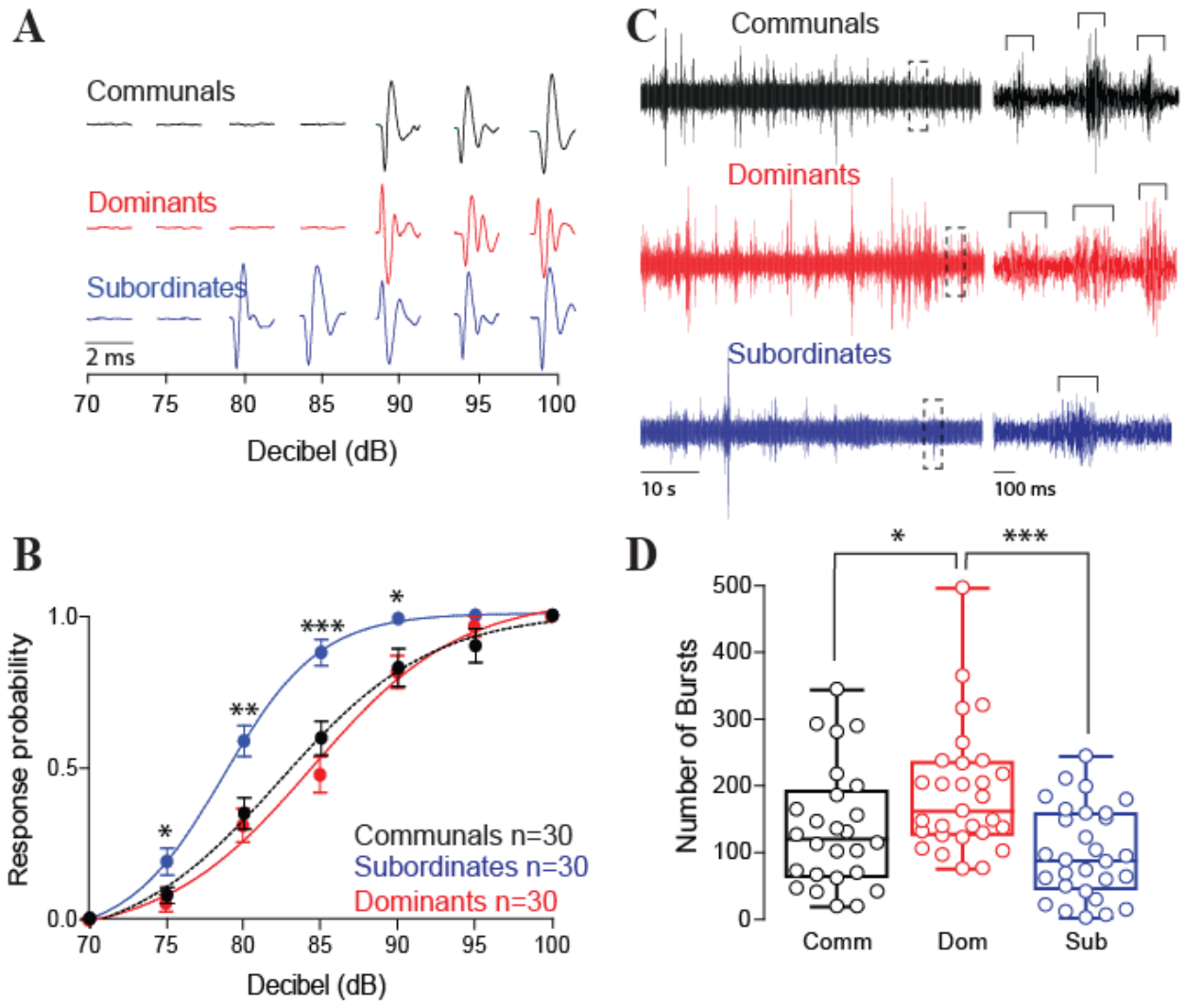


Figure 4 - Social status alters startle and swimming

A) Characteristic Mauthner escape potentials for the three social categories at each decibel level. B) Subordinate zebrafish have a lower threshold for the Mauthner startle response than both communals and dominants (significance markers compare subordinates to dominants). C) Representative 1-minute trace recordings of swimming activity for each social category (left); individual swim bursts in brackets (right inset). D) Box and whisker plots. Box encompasses 95% of data. Whiskers represent maximum and minimum values. Horizontal line represents median swim bursts over 1-minute recording period for each social group. Circles represent individual animals. Dominants swim at a higher frequency than communals and subordinates. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$.

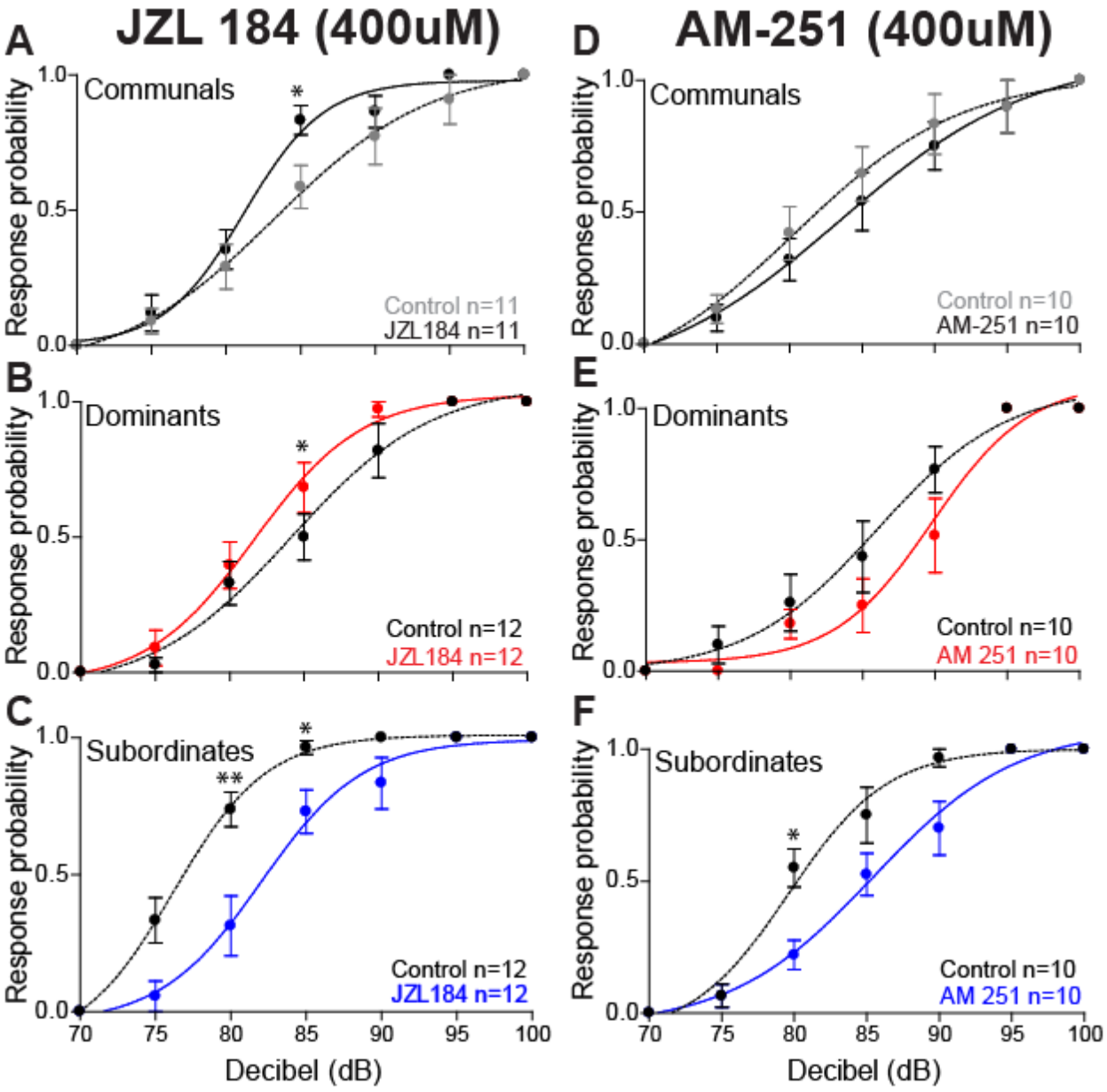


Figure 5 – JZL184 and AM-251 related ECS modulation of startle sensitivity in dominant and subordinate zebrafish

Augmentation of 2-AG with JZL184 affected the startle response differentially based on social status (A-C). Communal fish and dominants injected with JZL184 showed significant increase in startle at 85dB (A, B). In contrast, subordinates showed significant decrease is startle at 80dB and 85dB (C). Blockade of CB1 with AM-251 led to decreases in startle sensitivity across all social types (D-F), but only significantly decreased startle at 80dB in subordinates (F). * = $p<0.05$, ** = $p<0.005$.

Status-dependent influences of ECS modulation on swimming. Targeting ECS function also revealed status-dependent effects on swimming behavior. We used the same drugs to test the effects of ECS modulation on swimming activity.

JZL184 injection had similar status-dependent effects on swimming as it had on startle behavior. In communals, we observed no change in swim frequency (Figure 6A). However, we saw that JZL184 had opposite effects on swimming in dominants and subordinates. Dominants treated with JZL184 showed a reduction in swim frequency (Wilcoxon match pairs, $p=0.0322$; Figure 6B), while subordinates showed an increase in swim frequency (Wilcoxon match pairs, $p=0.0186$; Figure 6C). These results suggest that there are social status-dependent differences in ECS activity in the swim circuit, and that these differences can be partially reversed by treatments with JZL 184. We infer that the JZL 184 increased 2-AG levels (Long et al. 2009) and conclude that increasing 2-AG differentially affects activation of the swimming circuit depending on social status.

Blocking CB1 with 400μM AM-251 reduced swim frequency significantly in communals (Wilcoxon matched pairs, $p=0.0137$; Figure 7A), but had no effect on swimming in dominants (Figure 7B) or subordinates (Figure 7C). Thus, we were able to replicate previous findings that blocking the CB1 receptor reduces swim frequency in communal fish but were unable to demonstrate a robust difference in effects between dominant and subordinate fish.

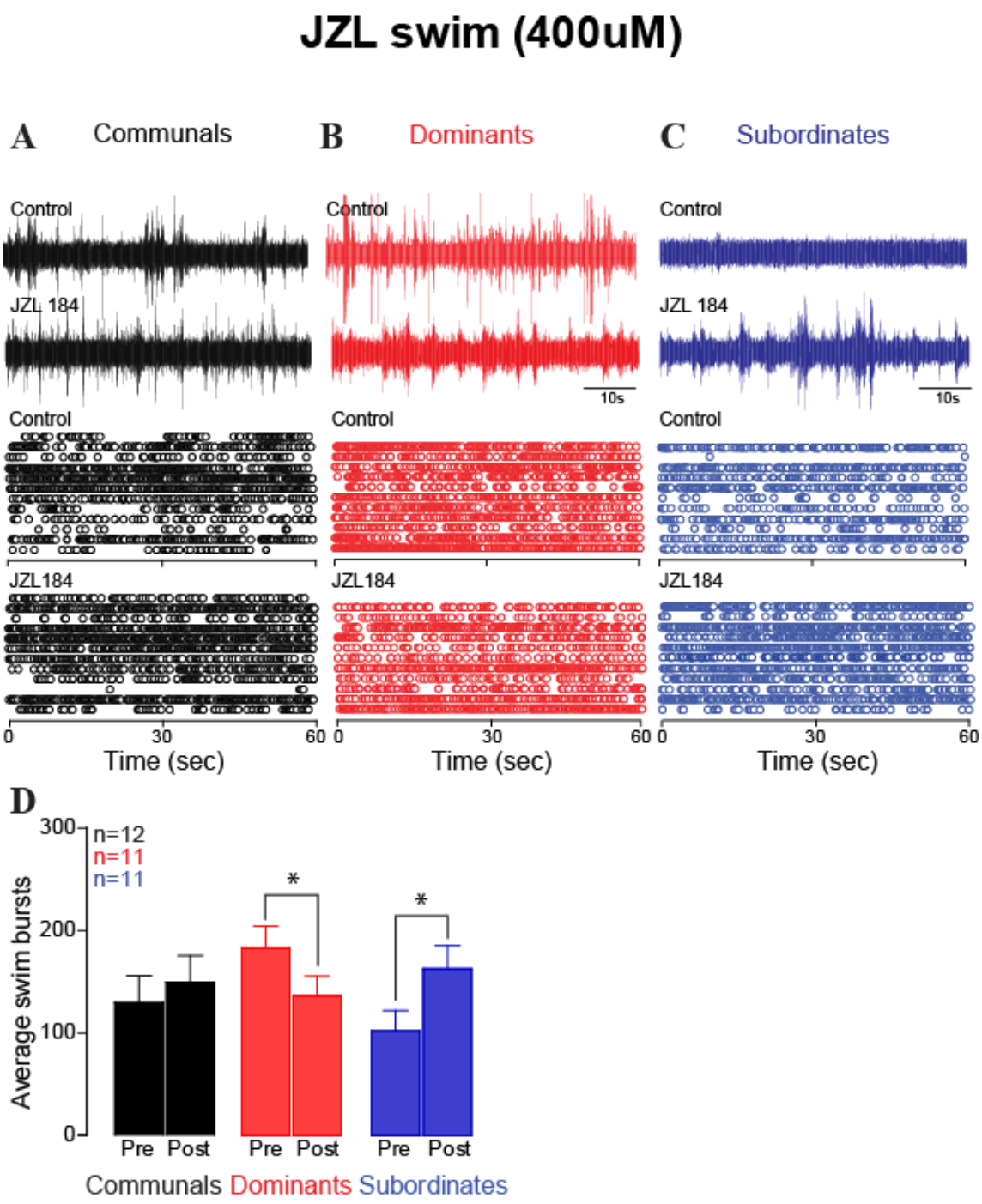


Figure 6 – Augmentation of 2-AG differentially affects swimming based on social status

Selected 1-minute individual trace recordings (top) and individual raster plots depicting swim bursts (bottom) for communal, dominant, and subordinate fish, before and after injection with JZL184 (A-C). Bar graphs of average swim bursts for control (n=12), dominant (n=11) and subordinate fish (n=11). JZL184 had no effect on swimming in communal fish, decreased swimming in dominants, and increased swimming in subordinates (D). * = $p<0.05$.

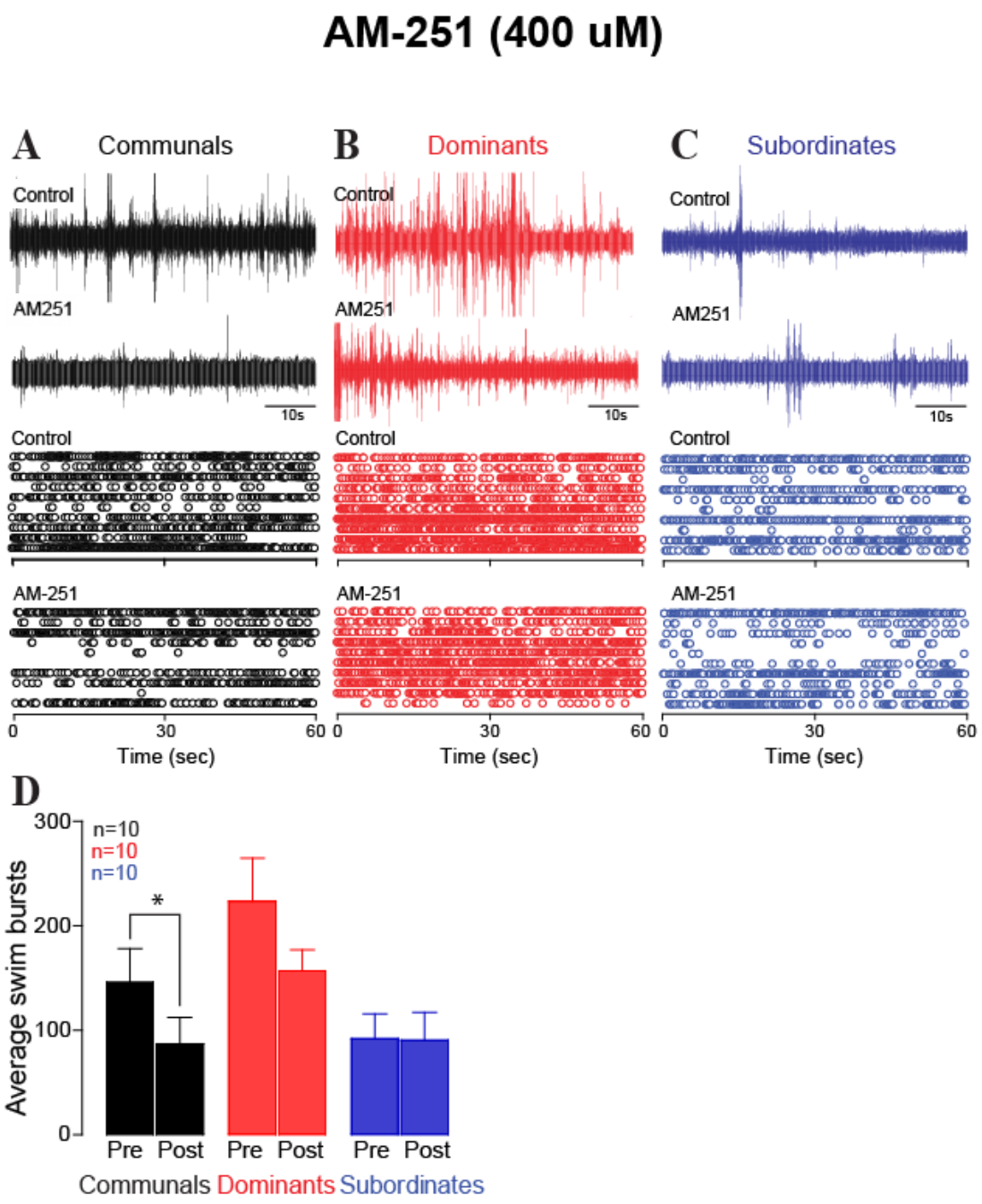


Figure 7 – CB1 blockade differentially affects swimming based on social status

Selected 1-minute individual trace recordings (top) and individual raster plots depicting swim bursts (bottom) for communal, dominant, and subordinate fish, before and after injection with AM-251 (A-C). Bar graphs of average swim bursts for controls (n=10), dominants (n=10) and subordinates (n=10). AM-251 decreased swimming in communal fish, decreased swimming in dominants (non-significant), and had no effect on subordinates (D). * = $p<0.05$.

Effects of two concentrations of AM-251 on motor behavior. We tested the behavioral effects of AM-251, a competitive inhibitor of the CB1 receptor, at two concentrations to determine if there was a threshold at which point AM-251 outcompetes 2-AG, and further to see whether that threshold differs between dominant and subordinate fish. We chose to test the effects of AM-251 at 100μM (low concentration) and 400μM (high concentration). We measured small differences in startle between the lower and higher drug concentrations (Figure 8). No significant differences in startle sensitivity were seen between the two concentrations in communals and dominants, however startle in subordinates was significantly reduced at 80dB only at the higher AM-251 concentration (Wilcoxon match pairs t-test, $p=0.0078$; Figure 8C2), suggesting that the lower concentration was insufficient to outcompete 2-AG for CB1 binding sites within the Mauthner circuit.

We found no significant difference between the effects of two AM-251 concentrations on swimming (Figure 9). Communals showed a similar decrease in swimming at both low and high concentrations. Dominants showed no significant change in swim frequency at either concentration. For subordinates, the near-significant reduction in swimming at the low concentration (Wilcoxon match pairs, $p=0.0781$) was eliminated at the high concentration ($p=1.00$). These results suggest possible differences in 2-AG levels and/or CB1 receptor concentrations between dominant and subordinate fish.

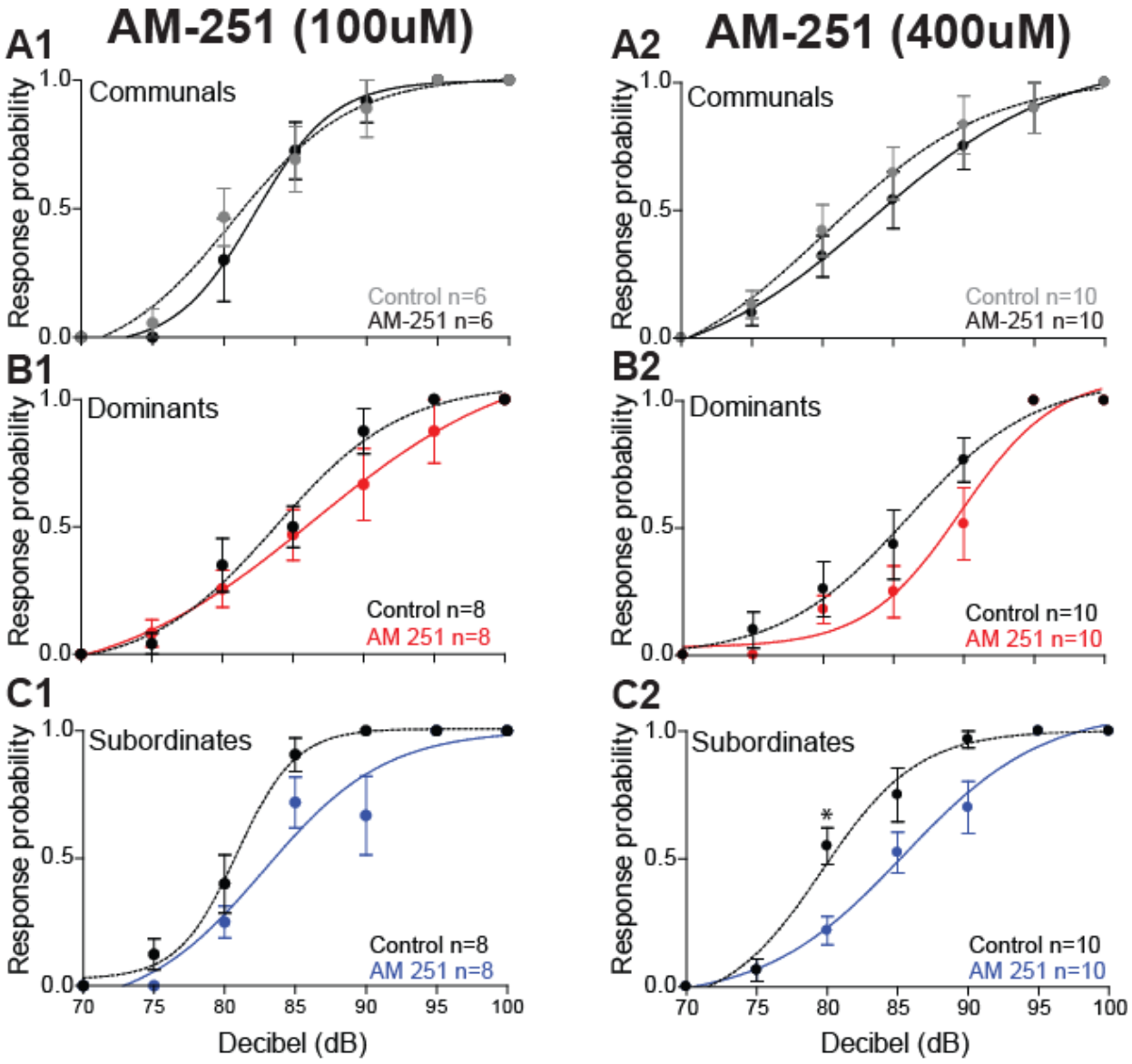


Figure 8 –Effects of two concentrations of AM-251 on startle behavior in zebrafish.

Startle response probability before and after injection with the CB1 receptor antagonist AM-251 at 100uM (A1-C1) and 400uM (A2-C2). Response probability is plotted as a function of sound intensity (dB) for communals (A), dominants (B), and subordinates (C). * = $p<0.05$.

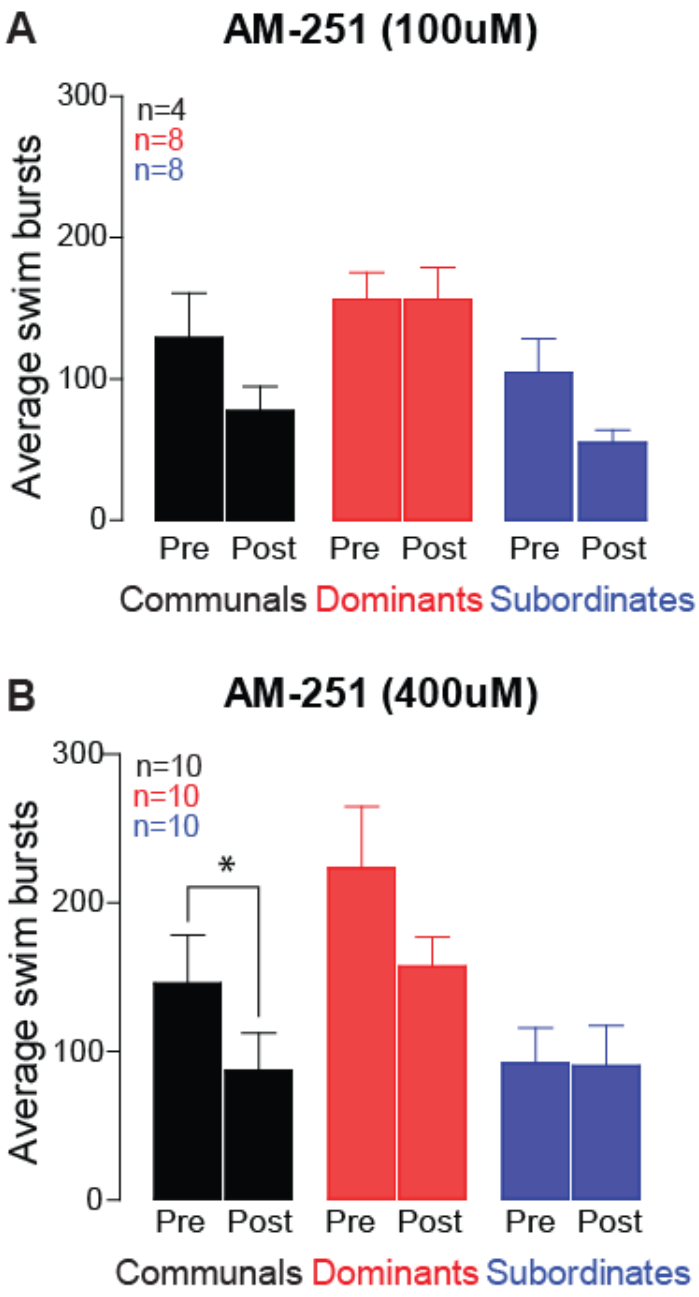


Figure 9 – Effects of AM-251 treatment on on swimming behavior in zebrafish.

Bar graphs of average swim bursts for the three social categories before and after injection of AM-251 at 100uM (A) and 400uM (B). * = $p<0.05$.

Interplay between the ECS and dopaminergic system in the regulation of motor behavior.

It is well known that the ECS and dopaminergic systems are highly interdependent (Cheer et al. 2007; Melis and Pistis 2007; Gardner 2005). Specifically, it has been shown that the dopamine 1 (D1) receptor can mediate the effects of ECS signaling on behavior (Zenko et al. 2011). Furthermore, dopamine is known to be involved in social regulation (Watanabe and Yamamoto, 2015), motivation (Hamid et al. 2016), and aggression (Filby et al. 2010). The most compelling evidence, for the purpose of our research, came from Cachope and colleagues (2007) who demonstrated in goldfish that 2-AG potentiates mixed synaptic transmission to the M-cell, and that this effect requires activation of the D1 receptor. Therefore, we decided to investigate the interactions between the ECS and the dopaminergic system in setting the balance in activation between the escape and swim circuits. We predicted that the effects of ECS modulation on startle and swimming were being mediated by dopamine. To test this hypothesis, we utilized a mutant zebrafish line in which the D1 receptor had been knocked out (D1KO).

Before any pharmacological manipulations were performed, we first tested the D1KO zebrafish behaviorally to determine whether they differed from WT communals. We found no behavioral differences between the WT and knockout fish (Figure 10). Next, we injected the knockout fish with JZL184 to determine its effects on startle and swimming behavior. We found no changes in startle sensitivity after drug injection compared to our pre-injection baseline (Figure 10A). This contrasted with our JZL184 injections in WT communals, in which we saw a significant increase in startle sensitivity (Figure 10B). Similarly, we found no change in swimming frequency in the D1KO animals following JZL184 injection (Figure 10C). These results suggest that the potentiating effects of JZL 184 injection on the M-cell startle response involve D1 receptor activity.

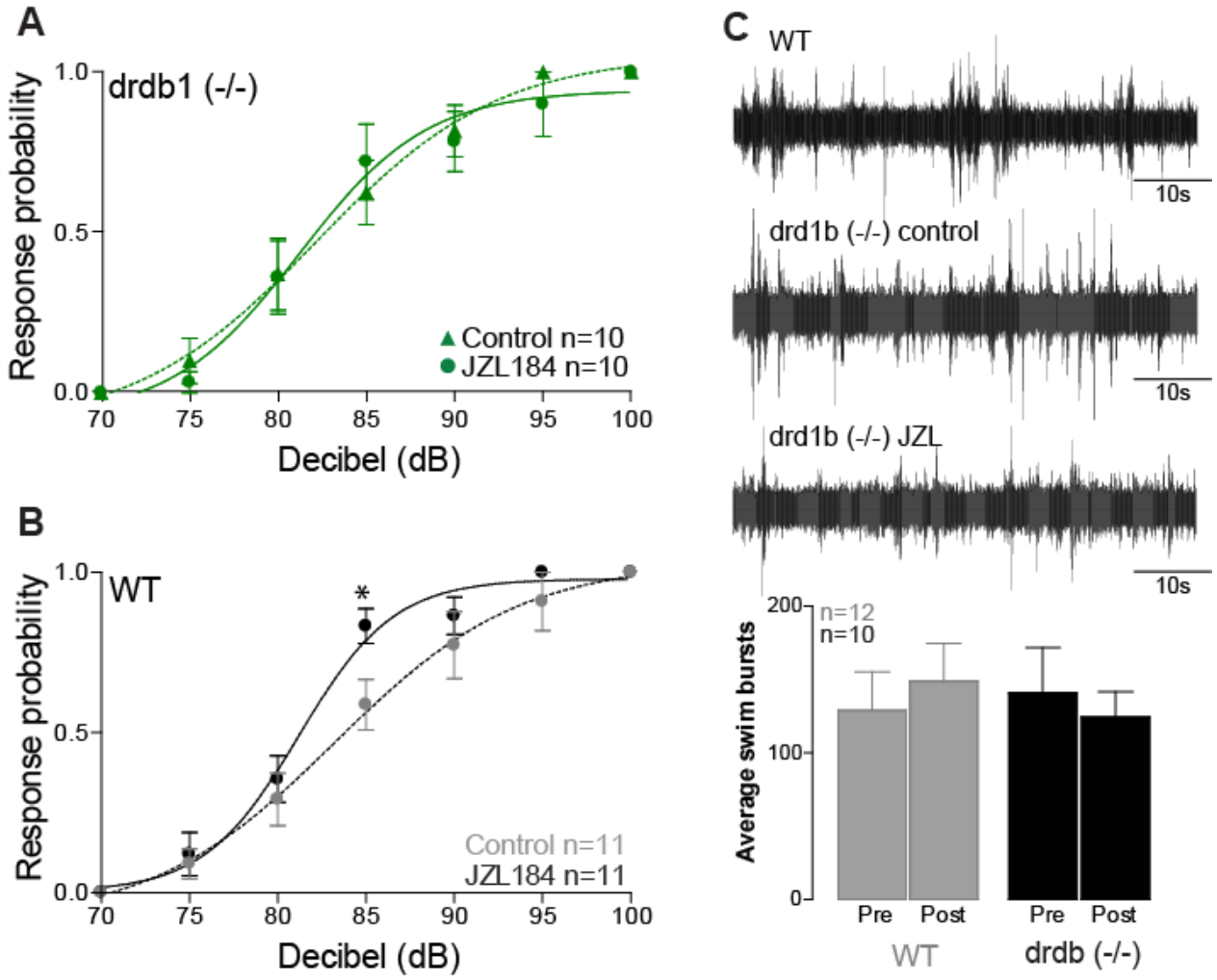


Figure 10 – Effects of JZL 184 treatment on drd1 KO zebrafish behavior

A) Response probability as a function of sound intensity, comparing D1KO zebrafish before (triangle) and after (circle) injection of JZL184. B) Response probability as a function of sound intensity for wild-type zebrafish before and after injection of JZL184. C) Above, 1-minute swim traces for wild-type zebrafish, and D1KO zebrafish before and after injection with JZL184. Below, bar graphs showing average swim bursts for wild-type zebrafish before and after JZL184 injection (gray), as well as D1KO before and after injection (black). * = $p<0.05$.

GENERAL DISCUSSION

The precise neural mechanisms underlying the social determination of behavior are poorly understood. A fresh avenue of inquiry into this subject was unveiled with the discovery that a class of lipophilic signaling molecules known as endocannabinoids plays a novel role in balancing the activation between competing motor circuits (Song et al. 2015). Furthermore, the endocannabinoid system (ECS) of vertebrates is remarkably sensitive to social influences (Morena et al. 2016). Collectively, this pointed to the possibility that the endocannabinoid 2-AG plays a role in shifting the balance in activation of motor circuits according to social status. Our findings that JZL184 led to a partial reversal of social status-dependent motor behaviors in both dominant and subordinate zebrafish provides evidence that the ECS plays a role in the neuronal control of social-status dependent control of swimming and startle response in zebrafish.

Endocannabinoids and the spinal locomotor circuit. We observed increased swimming activity in subordinate fish, and a surprising decrease in swimming activity in dominants, after JZL184 injection. The increased swimming that we observed in subordinates, and that Song and colleagues (2015) observed in communal zebrafish can be explained by the action of 2-AG in the CPG of the spinal locomotor circuit. CPG motor neurons receive inputs from ipsilateral e-INs and contralateral i-INs. In response to glutamatergic synaptic transmission from the e-IN, the motor neuron synthesizes and releases 2-AG, which then binds CB1 on the glycinergic i-IN terminals, inhibiting the release of glycine onto the motor neuron (Kettunen et al. 2005). Surprisingly, it was found that even when the GABA receptors on inhibitory inputs were blocked with strychnine, enhancing 2-AG still increased fictive swim frequency. This meant that 2-AG was not only inhibiting inhibitory

inputs but was also potentiating the inputs from excitatory interneurons onto the motor neurons. JZL184 injection prevents the degradation of 2-AG by MAGL and can result in increased 2-AG in the area around the motor neuron, and bind in greater quantity to CB1 on the i-IN. By diminishing inhibitory inputs and potentiating excitatory inputs onto motor neurons, 2-AG should increase swimming frequency. Our findings that JZL184 decreased swimming significantly in dominants is the opposite of expected effects based on the known excitatory actions of 2-AG on CPG activation (Song et al. 2015), and the knowledge that dominants swim more than subordinates (Figure 4D), we predict higher 2-AG levels in dominant fish. This prediction is further supported by the fact that augmenting 2-AG caused subordinates to mimic dominant-like swim frequency. Therefore, dominant fish should have elevated 2-AG pre-injection. Further increases in 2-AG would have negligible effects on the spinal locomotor circuit. Instead it could be primarily affecting higher brain regions such as the MLR, where it would inhibit descending inputs.

Endocannabinoids and the Mauthner escape circuit. Following JZL184 injection, we observed opposite effects on startle behavior based on social status. Startle sensitivity was decreased in subordinates and enhanced in dominants. In the startle circuit, it was found that 2-AG potentiates both the excitatory inputs to fast MNs (responsible for startle) and the inhibitory inputs to slow MNs (responsible for swimming). The net effect of 2-AG on this circuit is a strong activity-dependent potentiation of the escape circuit coinciding with a strong inhibition of the swimming circuit (Song et al., 2015). This is the "clutch-like" mechanism that allows a smooth transition from swimming, to startle, and then back to swimming (Song et al. 2015). It is possible that the inputs from the M-cell to MNs are socially regulated. However, it seems unlikely that these inputs would have significant effects on startle sensitivity because the M-cell is a command neuron. When

it fires, it elicits a startle. Neuromodulation of fast MNs downstream of the M-cell would have limited influence over whether or not the M-cell fires in response to an auditory stimulus because firing of the M-cell would be expected to override any downstream influences by 2-AG.

More importantly for the plasticity of the startle response, 2-AG modulates synaptic inputs to the M-cell. Specifically, 2-AG potentiates the mixed synaptic input from the VIIIth auditory nerve onto the M-cell. The VIIIth nerve, in addition to exciting the M-cell, also excites commissural and collateral interneurons that inhibit the M-cell. Weaker signals will preferentially activate the i-INs, and the M-cell will not fire. M-cell firing only occurs when the direct excitatory input from the VIIIth nerve is sufficient to override the indirect inhibitory inputs. (Korn and Faber, 2005).

It had been previously demonstrated that JZL184 treatment increased startle in communal zebrafish (Song et al. 2015). Our results supported these findings (Figure 5A). We found that JZL184 increased startle sensitivity in communals and dominants, but decreased startle sensitivity in subordinates (Song et al. 2015). All in all, JZL184 treatment negated behavioral status-dependent differences in the startle response of zebrafish. To better explain these findings, we have proposed a model of the possible interactions between the ECS and dopaminergic systems that affect M-cell activity (Figure 11). We further develop this model in a later section.

Our observation that JZL184 induces an elimination of status-dependent startle sensitivity could be explained by the large increases in 2-AG that ensue from inhibiting the degradative enzyme MAGL. Previous research demonstrated that JZL184 led to a more than 5-fold increase in 2-AG levels in murine brains (Long et al. 2009). If 2-AG levels vary based on social status, then the large increase in 2-AG would eliminate any differences in 2-AG concentration between dominants and subordinates that could be responsible for the original differences in startle

sensitivity. This would explain why JZL184 eliminated social status-dependent differences in startle sensitivity.

We also investigated the effects of a known CB1 receptor antagonist AM-251 on the startle response. We found that AM-251 (400μM) induced a general decrease in startle behavior across all social groups, although this effect only reached significance in subordinates (Figure 5D-F). These findings are difficult to interpret due to the contradictory conclusions reached by previous researchers. Working on zebrafish, Song and colleagues found that systemic injection of AM-251 decreased sensitivity of the auditory startle reflex (2015). These results seem to contradict findings by Cachope and colleagues – working on goldfish – who found that application of CB1 receptor antagonists (AM-251 and SR141716) had no effect on the amplitude of the excitatory post-synaptic potential (EPSP) from the VIIIth nerve onto the M-cell (2007). These researchers reasoned that 2-AG is not released tonically from the M-cell, and so blocking the receptor would not affect the startle response. Our finding, that blocking CB1 reduces startle sensitivity in subordinates, suggests either of several possibilities: 1) that the systemic application of AM-251 is blocking CB1 upstream of the M-cell, or 2) that there is some tonic release of 2-AG in the M-cell of subordinate fish. Considering the first possibility, blocking CB1 receptors on hair cells could affect the startle response by influencing sensitivity to sound. However, there is currently no evidence that hearing is influenced by ECS activity. The second possibility is supported by the higher startle sensitivity in subordinates and the known potentiating effects of 2-AG on startle behavior. However, this is complicated by the fact that JZL184, which is reported to increase 2-AG, also reduces startle sensitivity in subordinates. The findings by Song et al. are hard to reconcile with our results unless these researchers unintentionally selected subordinate or chronically stressed fish for their AM-251 experiments.

Effects of AM-251 at low and high concentrations on motor behavior. We found that subordinate swimming behavior appeared to be more sensitive to lower doses of the CB1 receptor antagonist AM-251, while dominant swimming was unaffected at either concentration (Figure 8). Concentration-dependent effects of neuromodulators on swimming frequency have previously been reported. Clemens et al. (2012) found that, in a reduced spinal cord preparation of the *Xenopus laevis* tadpole, bath application of dopamine differentially affected locomotor frequency based on concentration. The low concentration reduced the occurrence of bursts and fictive swimming episodes while increasing episode cycle period. Conversely, the high dopamine concentration increased burst and swim episode frequency while decreasing episode cycling period. These concentration-dependent effects appeared to result from the balance between the activities of D1 and D2 dopamine receptors. The inhibitory D2 dopamine receptors have a higher binding affinity, so are saturated at lower concentrations – explaining the reduced swim frequency. However, at higher dopamine concentrations, D2 receptors are completely saturated and D1 receptors become increasingly recruited, thus increasing swim frequency (Clemens et al. 2012). The concentrations-dependent effects of the CB1 antagonist AM-251 cannot be explained by this logic, however, since CB1 is the primary cannabinoid receptor in the brain. While CB2 is present in the brain at much lower quantities, it is also an inhibitory receptor, so its activation would not be expected to have different effects from CB1 regardless of its binding affinity.

Our findings that blocking the CB1 receptor had concentration-dependent effects on dominants versus subordinates could be explained by the competitive binding of AM-251. It competes with 2-AG for CB1 binding sites. AM-251 (Ki = 7.5nM) has a much higher affinity for CB1 than does the endogenous agonist 2-AG (Ki = 58.3nM) (Pertwee et al. 2010). It is possible that dominant fish have higher levels of spinal 2-AG than subordinates. This prediction is based

on the known effects of 2-AG in potentiating swimming activity (Song et al. 2015), and the knowledge that dominants swim more than subordinates (Figure 4D). Higher spinal 2-AG would explain why a higher concentration of the competitive antagonist AM-251 is needed to impact swimming in dominants. 2-AG and AM-251 compete for CB1 receptor binding sites, so higher AM-251 concentration would be needed to compete with higher 2-AG concentrations.

Based on previous studies where ECS modulation was used to probe behavior, it is not surprising that the interplay between the ECS and social status in the control of behavior can depend on the concentration of AM-251. One study in rats found that the CB1 receptor agonist HU-210 had anxiolytic effects at low doses but induced anxiety-like states at higher doses (Hill and Gorzalka 2004). Moreover, the researchers found that mice subjected to chronic unpredictable stress reacted to the lower dose in the same way as unstressed rats reacted to the higher dose – by developing anxiety-like behavior. This finding is in line with our results that subordinate fish, which are subjected to chronic stress, are more susceptible to ECS modulation. The findings from Hill and Gorzalka suggest that chronic stress leads to a sensitization of the ECS; but seeing that there is no evidence of increased CB1 in stressed animals, they suggest that the induction of anxiety-related behavior is being induced by altered expression of downstream effectors such as the κ-opioid receptor. Due to the interconnections of the ECS with other neural systems in the control of behavior, it can be difficult to dissect the effects of the ECS in isolation.

Involvement of D1 receptor in mediating effects of ECS modulation on behavior. Our results from experiments with D1KO zebrafish suggest that removing D1 receptor functionality eliminates the enhancement in startle induced by augmenting 2-AG through treatment with JZL184 (See Figure 10A). Previous research examined the interplay between dopamine (DA) and

the ECS in an *in vivo* goldfish preparation (Cachope et al. 2007). These researchers found that pretreatment with a D1 antagonist prevented enhancement of M-cell excitability by a CB1 receptor agonist. Rather than using an *in vivo* preparation we aimed to show that these effects could be replicated in freely behaving animals. Our experiments in which we measured startle sensitivity show a clear difference between the effects of ECS modulation on D1KO communals (Figure 10A) and WT communals (Figure 10B). Whereas previous research focused exclusively on the escape circuit, we also considered the interplay between these systems in the regulation of swimming. We observed a modest reduction in swim frequency after JZL184 treatment in D1KO. Contrastingly, in WT communals JZL184 induced a modest increase in swimming (Figure 10C). Although not significant, these results suggest an association between the dopaminergic and ECS system, at least as concerns modulation of swimming behavior. The results also provide justification for further research into the interplay between dopamine and the ECS in the regulation of swimming. Overall, these results point to the role played by the dopaminergic system in mediating the effects of the ECS on status-dependent changes in motor behavior.

Discussion summary. JZL184 injection led to a partial reversal of social-status dependent activation of motor circuits in both dominants and subordinates. We were able to replicate findings from previous startle experiments by Song and colleagues, in that JZL184 injection led to an increase in startle in both communal and dominant animals. We also replicated their findings that JZL184 induced increased swimming frequency. After JZL184 injection, we observed no change in communal swimming but saw significant increase in swimming in subordinates. We also discovered social status-dependent effects of ECS modulation, in which JZL184 had opposite effects on dominants and subordinates. Our results support previous observations of the role the

ECS plays in modulating the pattern of activation of the escape and swim circuits and extend on those findings in demonstrating the importance of social factors in regulating ECS and motor behavior.

We also examined the effects of a CB1 receptor antagonist on motor behavior and replicated findings by Song and colleagues, in that blocking CB1 led to modest decreases in dominant and communal startle behavior and significant decreases in startle behavior in subordinates. Moreover, we replicated their findings that blocking CB1 reduced swimming frequency in communal fish. We also found that swimming was reduced to a greater degree at the lower AM-251 concentration than at the higher concentration, suggesting that the effects of AM-251 depend on its concentration. These effects could indicate the presence of an additional cannabinoid receptor subtype with an opposite functional valence and lower affinity for AM-251. If this was true, higher concentrations of AM-251 would begin binding to the hypothetical receptor, off-setting the inhibitory action of CB1 receptor binding on swimming. Such a scenario would explain why the lower AM-251 concentration had a greater effect on subordinate swimming than the higher concentration. Finally, we replicated previous findings by Cachope and colleagues suggesting that the D1 receptor is involved in the modulatory effects of the ECS on startle behavior.

Proposed model for social regulation of the M-cell. We have found that treatment of fish with JZL184 partially reverses socially-mediated activation of the startle and swimming behaviors in zebrafish. The MAGL inhibitor, JZL184, had opposite effects on both swimming and startle depending on the social status of the fish. Compared to their pre-injection baseline behavior, dominants injected with JZL184 had a more sensitive startle response and reduced swim frequency

(Figure 4B). On the other hand, subordinates treated with JZL184 had a less sensitive startle response and greater swimming rate compared to baseline (Figure 4C). Moreover, our findings suggest that the dopaminergic system mediates the behavioral effects of 2-AG in freely behaving animals. Together, our findings suggest that the ECS is a key regulator of social status-dependent behavior, and that dopaminergic modulation of the M-cell is involved in the regulatory effects on startle sensitivity.

Work done by Cachope and colleagues (2007) showed that CB1 receptors are closely associated with dopaminergic terminals near the M-cell. This suggests that these dopaminergic terminals express CB1 receptors. They also found that the activation of the group 1 metabotropic glutamate receptor (mGluR1) led to a lasting potentiation from the VIIIth nerve onto the M-cell. They inferred that 2-AG was synthesized and released from the M-cell in response to mGluR1 activation, and that the lasting potentiation they observed required 2-AG binding to CB1. They came to this conclusion based on an experiment showing that the potentiating effects of mGluR1 activation on the M-cell were eliminated by local application of a CB1 receptor antagonist. Moreover, they showed that 2-AG increased M-cell excitability by potentiating the release of DA from nearby DAergic cells. Binding of DA to D1 receptors on the M-cell increases PKA activity, which is responsible for the potentiation of both electrical and glutamatergic synaptic transmission onto the M-cell. Based on these findings they developed a model in which retrograde 2-AG signaling mediated by dopamine potentiates the mixed synapse of the VIIIth nerve onto the M-cell lateral dendrite. We elaborated on this model to explain the status-dependent effects of ECS modulation on the startle response (Figure 11).

Our model is dependent on three primary inputs to the M-cell: DAergic, GABAergic, and the mixed club ending of the VIIIth nerve (glutamatergic and electrical). All three types of inputs

are known to innervate the lateral dendrite of the M-cell (Korn and Faber, 2005). It is important to keep in mind that CB1 activation affects DA cells differently from other cell types in that it potentiates release of dopamine, whereas it inhibits release of other neurotransmitters (Cachope et al. 2007).

Our model predicts that dominants primarily activate the neurochemical pathway leading from DAergic to GABAergic inputs (Figure 11A). Release of DA would then lead to greater release of GABA onto the M-cell, resulting in higher threshold for M-cell firing. By increasing 2-AG we would be shifting activation to the direct pathway from the DA cell to the M-cell. This is based on our model's prediction that the indirect pathway from DA cell to GABA cell is primarily active in dominants, but that upregulating 2-AG shifts activation to the direct pathway (from DA cell to M-cell), which promotes a reduction in startle threshold, mimicking subordinate behavior.

In subordinates, our model predicts that 2-AG induces high excitability of the M-cell by binding to CB1 on DA neurons and potentiating release of DA onto the M-cell (Figure 11B). DA then binds to D1 on the M-cell and activates the cAMP- and PKA-dependent pathway that potentiates the mixed synapse and enhances M-cell excitability. This enhanced excitability causes subordinates to have a more sensitive startle response. When 2-AG is increased with JZL184, increased DA is released which activates the D1 receptor on nearby GABAergic neurons. This induces the release of GABA onto the M-cell, which precludes the excitatory effects of M-cell D1 receptor activation and ultimately reduces M-cell excitability.

This model, while preliminary, sets the stage for future experiments to further elucidate the interplay between the ECS and dopaminergic system in the establishment of socially-determined behavior. Below we have outlined future avenues to be pursued.

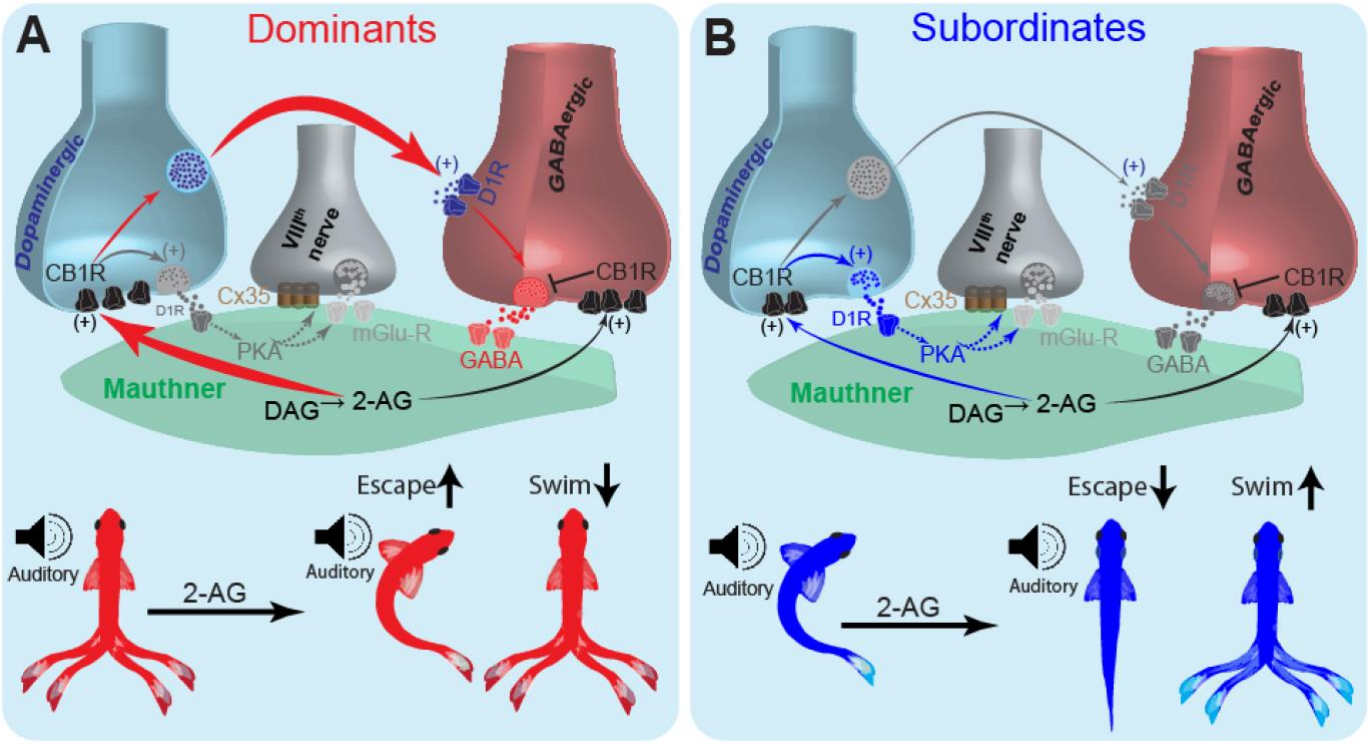


Figure 11 - Proposed model of ECS interaction with dopaminergic system at the M-cell

Schematic model for social status-dependent regulation of neurochemical inputs to the M-cell: The M-cell (green) receives inputs from DA cells (blue), the excitatory VIIIth cranial nerve (gray), and inhibitory GABA cells (red). Our model predicts distinct neurochemical pathways in dominants (A) and subordinates (B) responsible for differences in startle sensitivity. These pathways are proposed based on differential effects of JZL184 treatment on startle behavior (bottom). Higher baseline 2-AG in dominants is responsible for activation of the "inhibitory pathway" via GABA release. Lower baseline 2-AG in subordinates activates the lower threshold "excitatory pathway" via the VIIIth nerve, responsible for higher startle sensitivity.

Future experiments. Future experiments should aim to determine whether DAergic neurons express CB1, and if not, by what mechanism the ECS interacts with the dopaminergic system. One useful experiment would be to stain for CB1 in the diencephalic posterior tubercular nucleus (PTN), which provides descending dopaminergic innervation in fish (Ryczko and Dubuc 2017). Assuming DAergic neurons in the PTN project to the M-cell, we would anticipate CB1 mRNA to be present in the PTN, while the CB1 receptors would be present on DA terminals in the hindbrain. Thus, *in situ* hybridization should be performed to test for CB1 mRNA in the PTN and immunohistochemistry to test for CB1 in the hindbrain.

Previous work by Cachope and colleagues (2007) provided evidence that the ECS can act in a non-canonical way to potentiate synaptic transmission by acting through the dopaminergic system. These researchers demonstrated co-localization of CB1 with DAergic neurons. However, further experiments are needed to ensure that the CB1 is on the DA cells themselves and not on processes immediately adjacent. Additionally, the source of the DA fibers is unknown, although the PTN is the primary source of dopamine in basal vertebrates (Ryczko and Dubuc 2017). To the best of our knowledge the diencephalic PTN has never been stained for CB1, and this would provide a more conclusive answer to whether dopaminergic inputs to the M-cell express this receptor.

Next, continued research should be done on the D1KO zebrafish line to determine (1) whether they can form stable social relationships, (2) whether the swimming and startle of D1KO dominants and subordinates differs from that of their wild-type counterparts, and (3) whether JZL184 injections will achieve a reversal of socially-determined behavior. If the transgenic fish are capable of forming stable relationships but do not express socially appropriate motor behaviors, then the D1 receptor would be deemed necessary for the expression of social status-dependent

motor behavior. If they form stable relationships, but are unaffected by JZL184, then this would support our theoretical model of status-dependent modulation of M-cell inputs (Figure 11).

Another avenue to explore is the effects of ECS modulation on aggression. In mice, augmentation of 2-AG with JZL184 eliminated aggressive behavior and increased attacks by other mice (Aliczki et al. 2015). The reduced aggressiveness characteristic of subordinates could be due to higher levels of 2-AG. To test this, JZL184 injections would be performed on isolated fish just prior to pairing. One fish will be injected with JZL184, while the other is injected with saline. We predict that the JZL184-injected fish would be more likely to become subordinate. This experiment would complement our present research by rounding out the behavioral effects associated with either activation or inactivation of the ECS, i.e. we would then know whether the reversal in behavior we observed as a result of 2-AG enhancement was an actual role reversal, or only the reversal of motor behaviors associated with a particular social status.

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APPENDIX: IACUC APPROVAL LETTERS

East Carolina University
Tomorrow starts here.

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834-4354

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

November 7, 2017

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 #D320a) was reviewed by this institution's Animal Care and Use Committee on November 7, 2017. The following action was taken by the Committee:

"Approved as submitted"

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