DOPAMINE MODULATION OF ESCAPE MOVEMENTS IN LARVAL ZEBRAFISH

HUMBOLDT STATE UNIVERSITY

By

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ABSTRACT

Dopamine modulation of escape movements in larval zebrafish

Gabrielle Sturchio

Dopamine is a neurotransmitter involved in the control of motor coordination. Its modulation of inhibitory and excitatory processes in the motor pathways of the brain and spinal cord influences initiation and control of movement behaviors. This study examined the effects of dopamine on the physiological responses of a pair of hindbrain neurons, called the Mauthner cells, and the associated behavioral effects, in larval zebrafish. The Mauthner pair coordinate swift escape responses to sensory stimuli in zebrafish. Evidence from previous studies suggests that the Mauthner neuron receives input from dopamine neurons in the brain, and my experiments were to determine whether dopamine actually modulates Mauthner cell activity. Physiological recordings of the Mauthner and other descending neurons were done using intracellular calcium imaging, with an escape-eliciting tap stimulus applied during cell recording. The effects of bath-applied dopamine on Mauthner neuron responses were assessed by comparing responses before, during, and after dopamine treatment in the same larvae (within subjects design), and, in separate experiments, by comparing responses in dopamine treated larvae and untreated control larvae. Dopamine exposure produced increases in Mauthner calcium response magnitude and likelihood of behavioral response to stimuli. These differences were not found in the control experiments. In the context of this and previous research, dopamine appears to facilitate escape-related swimming in larvae while inhibiting spontaneous swimming activity.

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Dopamine modulation of escape movements in larval zebrafish

The amine neurotransmitter dopamine (DA) is well known as one of our "pleasure chemicals" and part of the brain's reward circuit, but actually assumes a much wider range of responsibilities in the body. These include motor activity, feeding, and cognition, as well as emotion and motivation. Of interest to this study is dopamine's role in movement.

In humans, dopamine imbalance is involved in the motor deficits of neurological disorders such as Parkinson's and Huntington's diseases. The proposed mechanisms for Huntington's, in particular, are imbalances in excitatory and inhibitory transmissions that lead to a disconnection between brain structures, thereby disrupting pathways of information throughout the brain. The nigrostriatal DA pathway is implicated in a motor dysfunction called chorea, which causes abnormal involuntary movements; a general over-activation of dopamine accompanies this symptom. Loss of DA inputs can lead to akinesia, a loss of movement characteristic of Parkinson's disease and later stages of Huntington's disease (Andre, Cepeda, & Levine, 2010). In Parkinson's disease, one of the major motor pathologies is a loss of dopaminergic neurons in the substantia nigra and a steep drop in striatal dopamine levels (Pienaar, Gotz, & Feany, 2010). Dopamine's role in these disorders appears to be to balance the excitatory effects of the neurotransmitter glutamate (Starr, 1995). Parkinson's disease is proposed to be a glutamate hyperactivity disorder, and DA released from nigrostriatal nerves is able to shift the glutamate balance

in the basal ganglia between hyperactivity and hypokinesia in mammals. This influence affects glutamatergic and GABAergic innervation of two functionally opposed pathways through the basal ganglia to the thalamus: the direct path which facilitates movement and the indirect path which inhibits movement. The two pathways have different types of DA receptors by which dopamine increases activity through the direct path and decreases it through the indirect path (Redgrave et al., 2010). Without proper DA functioning in these pathways, conscious control over movement diminishes. As such, the balance between glutamate and dopamine in key areas of the basal ganglia is critical for transmitting sensory information to motor networks and initiating appropriate movement responses (Andre, Fisher, & Levine, 2011; Johnson & Napier, 1997; Starr, 1995). Restoring this balance is proposed to be useful in treating some of these symptoms.

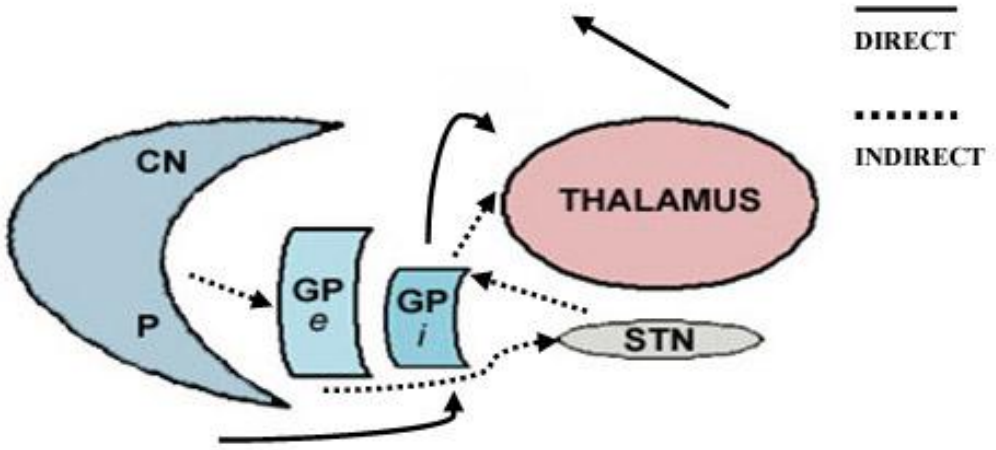


Figure 1. Motor signals follow two paths through the mammalian basal ganglia: the direct pathway which facilitates movement and the indirect pathway which inhibits movement. Dopamine is involved in the modulation of both.

Of course, the basal ganglia are not the only brain structures that are essential for the initiation and coordination of motor behaviors, nor are they the only structures that are modulated by dopamine. The spinal cord, for example, can be both activated and regulated by dopamine exposure (Barriere, Mellen, & Cazalets, 2004). Efforts toward treatment for motor disturbances also motivate research on descending neurons that project to the spine. The task of descending neurons is to communicate the brain's motor commands to the spinal cord. From the spinal cord, muscle contractions are initiated and movement occurs (Orger, Kampff, Severi, Bollmann, & Engert, 2008). Animal studies of these descending neurons are a promising way to map locations and connections of neural networks involved in motor control. The anatomical and physiological knowledge gained from such studies in "lower" vertebrates like fish provides a blueprint for studying the more crowded and complex motor control networks in the human brain.

In zebrafish, dopamine activity is involved in the coordination and frequency of movement behaviors (Thirumalai & Cline, 2008) and can follow a similar pattern of degeneration as in human motor disorders (Xi et al., 2010). Zebrafish are popular subjects for the study of spinal projection neurons because their hindbrains have similar structure and function to other vertebrates, including humans, but also have a smaller and therefore more manageable number of neurons. There are about 220 descending neurons total in larval zebrafish (Gahtan, Sankrithi, Campos, O'Malley, 2002), all of which are individually identifiable (Kamali et al., 2009). One motor similarity between zebrafish and higher vertebrates is that they have an optomotor reflex (OMR), which is a reflex to

turn and move in the direction of visually perceived motion. This suggests that visuomotor pathways are organized similarly in zebrafish and other vertebrates, including primates (Orger, Smear, Antis, & Baier, 2000). Young zebrafish are almost entirely transparent as larvae, making neural bundles visible to the naked eye. The notochord, which will later develop into the spinal column, is also visible under a light microscope. These characteristics make neuro anatomy and brain function much easier to study than it is in higher vertebrates. Zebrafish develop quickly, and externally rather than in a womb, so development is also accessible to study. Larvae, because they have only been alive for several days, are more likely to rely on hard wired neural circuits rather than on experience to respond to their environment (Gahtan & Baier, 2004), an advantage in studying the cellular basis of behavior. Zebrafish are also easily genetically modified, but that particular advantage will not be utilized in this study.

Zebrafish have a known motor escape circuit in the hindbrain that relies on specific cells to orchestrate movement. The Mauthner cell is a descending neuron in the fish hindbrain that projects down the spine and sends signals to motor neurons. It is one of the only descending neurons whose connectivity is known (Sankrithi & O'Malley, 2010). The Mauthner cells, which come in a pair, help to orchestrate fast escape movements (Liu & Fetcho, 1999). One Mauthner fires a single action potential in response to an escape-eliciting stimulus, exciting motor neurons on the opposite side of the body, causing the fish to make a swift and automatic C-bend movement that propels it away from the offending stimulus (Eaton, Lee, & Foreman, 2001; O'Malley, Kao, &

Fetcho, 1996). The Mauthner has homologous partner cells in consecutive segments, MiD2 and MiD3, which cooperate at varying levels depending on the type of stimulus. All three cell types in the escape network are activated by rostral stimuli to the head, resulting in the strongest C-bend, while only Mauthner is activated by caudal stimuli to the tail (O'Malley et al., 1996). While it is certainly the coordinator, the Mauthner is not entirely responsible for escape commands; C-bends can still be performed in the absence of the Mauthner cell and its segmental homologs, but these responses take approximately twice as long to occur (Gahtan et al., 2002; Liu & Fetcho, 1999). Because of the Mauthner cell's distinct, easily identifiable appearance and its known specific function and connectivity, it is a popular reticulospinal neuron to study.

The Mauthner cell and its homologs are surrounded by cells and neuropil that show strong reactivity to immune markers for both dopamine and serotonin (McLean & Fetcho, 2004). The distribution of this amine reactivity suggests an influence on both the inputs and outputs of these descending neurons. What remains to be tested is whether amine neurotransmitters modulate physiological responses of Mauthner array neurons and actual motor responses. This study will seek to specify the effects of dopamine on the physiological responses of the Mauthner cell and escape response of larval zebrafish.

Literature Review

Dopamine and Motor Induction and Disruption

Dopamine and its agonists can activate and modulate the motor networks of the mammalian spinal cord, with effects that last up to several hours. Bath application of DA induces slow rhythmic "fictive activity" and consistent bursts of action potentials in the isolated lumbosacral spinal cord of rat pups, when bath-applied to the ventral root (Barriere et al., 2004).

Dopamine seems particularly relevant to regulating swimming behaviors. In the leech nervous system, for example, direct application of dopamine to descending neurons disrupts swimming signals and prevents the signals from first being initiated, but does not have this inhibitory effect on crawling signals (Crisp & Mesce, 2004).

In zebrafish larvae whose dopamine neurons have been genetically disrupted, motor coordination suffers (Xi et al., 2010). Larval *pink1* mutants, in which the *pink1* gene is knocked down, have altered positioning and patterning of DA neurons in the ventral diencephalon, an area involved in motor coordination and implicated in human disorders such as Parkinson's. Normal zebrafish larvae are capable of escape swimming at 2 days post-fertilization (dpf) and show robust spontaneous swimming by 5dpf (Thirumalai & Cline, 2008). In contrast, most mutant larvae are either unresponsive or barely responsive to tactile stimuli at 3dpf. This lack of response can be corrected by repairing the altered mRNA sequence or by applying a D1 dopamine receptor agonist. Mutants also do less free movement at 5dpf than unaltered larvae, swimming slower,

shorter distances, with decreased coordination, and hugging the sides of the dish rather than crossing the open space in the center (Xi et al., 2010). In zebrafish larvae, the mutation is associated with a loss of dopamine neurons and misplaced projections in the ventral diencephalon. *PINK1* mutations are also sometimes witnessed in human Parkinson's disease, with a loss of DA neurons in the substantia nigra.

Exogenously applied dopamine, such as the bath solution which will be used in this study, decreases the amount of spontaneous swimming that zebrafish larvae do at 5dpf. Motor neurons that show spontaneous spiking in control saline solution do not have any spontaneous activity during dopamine exposure at this age. At 3dpf, spontaneous swimming episodes cease completely during dopamine exposure, though movement can still be elicited with stimuli. These effects are removed by washing out the dopamine solution (Thirumalai & Cline, 2008).

Consistent with these results, increases in dopamine release achieved with reuptake inhibitors and receptor antagonists also affect swim episodes. Blocking DA reuptake with bupropion hydrochloride prevents any swimming episodes, while inhibiting DA with D2-receptor antagonists results in more swimming episodes than in the control condition (Thirumalai & Cline, 2008).

In human patients, drug-induced motor disorders are a fairly common side effect of dopamine-blocking antipsychotic medications (Caligiuri, Jeste, & Lacro, 2000; Caligiuri et al., 2009). These drugs target D2 receptors and block dopaminergic activity in nigrostriatal pathways as well as the frontal cortex where they are intended to treat

disorders such as schizophrenia. Acute drug-induced conditions such as Parkinsonism and dystonia usually recede as the patient develops a tolerance to the medication, but permanent damage to motor systems can also occur. Tardive dyskinesia, a condition of abnormal involuntary movements that persists over time and is irreversible in some cases, may be due to loss of neurons in the basal ganglia; the antipsychotic haloperidol kills dopaminergic neurons in the substantia nigra (de Jesus Mari et al., 2004). In contrast, dopamine agonist drugs used in the treatment of Parkinson's symptoms sometimes induce temporary psychotic symptoms in patients (Stefanis et al., 2010) which are alleviated with discontinued use.

Dopamine Modulation of Motor Systems

Dopamine modulates induced motor activity in the spinal cords of rats. Bath-applied DA significantly increases burst amplitude and duration, but also slows down and stabilizes the motor rhythms (Barriere et al., 2004). Dopamine has a long-term effect on motor rhythms that isn't achieved using serotonin, another amine neurotransmitter involved in central motor signaling and modulation (McLean & Fetcho, 2004).

In live anesthetized rats, dopamine modulates both excitatory and inhibitory processes in the ventral pallidum, an area near the basal ganglia implicated in both schizophrenia and Parkinson's disease as a site of glutamate over-activity. Direct drug application changes the action potential firing rate of a majority of ventral pallidum neurons. A majority of the inhibitory GABA-responsive neurons tested show a decrease in their inhibitory response when exposed to DA, and a majority of the excitatory

glutamate-responsive neurons have a decrease in excitatory response. In neurons that are sensitive to both GABA and glutamate, DA modulates the responses to both (Johnson & Napier, 1997).

In the spinal cords of adult lampreys, a primitive jawless fish, dopamine modulates the synaptic transmission between reticulospinal neurons and spinal motor neurons. Reticulospinal neurons make up the main descending motor system of these animals. Their modulation is presynaptic, as evidenced by the effect on the chemical component of glutamatergic excitatory postsynaptic potentials (EPSPs) and not the electrical component. During stimulation of reticulospinal axons, bath-applied (exogenous) DA reduces the amplitude of the chemical aspect of glutamatergic EPSPs, without any effect on resting membrane potential. Application of bupropion, a dopamine reuptake blocker that increases the (endogenous*)* concentration of extracellular DA, also reduces the amplitude of monosynaptic EPSPs. Both of these effects can be recovered or partially recovered after dopamine wash-out. Dopamine modulates this system by suppressing glutamate release from reticulospinal neurons to motor neurons (Svensson, Wikstrom, Hill, & Grillner, 2003).

Dopamine and the Mauthner Cell

Dopamine's effect on the Mauthner cell has been studied in goldfish (Pereda, Triller, Korn, & Faber, 1992). Dopaminergic fibers are densely distributed in the synaptic bed around the Mauthner's dendrites, though they don't make direct contact with the cell or its presynaptic terminals. Application of dopamine directly above the

Mauthner's lateral dendrite enhances both the chemical aspect and the electrical coupling aspect of the cell's mixed EPSPs, and decreases the cell's input resistance, when activated by stimulation at the eighth nerve and spinal cord inputs. These effects last up to 90 minutes, beginning after about 5 minutes of bath application. Effects are blocked by a D1 receptor antagonist, indicating D1 type dopamine receptors are probably responsible for controlling this type of response. Dopamine may act on the Mauthner cell by increasing electrical conductance and chemical transmission, and its actions on these appear to be independent. Replication of this study (Pereda, Nairn, Wolszon, & Faber, 1994) suggests that dopamine usually modulates the Mauthner postsynaptically, and supports the finding that it increases both gap junction conductance (electrical coupling) and activation of glutamate receptors (chemical transmission) involved in the Mauthner EPSPs.

The diversity of these effects on motor coordination presents a need for further research and more specific details of which structures and processes are modulated by dopamine, and how. With dopamine controlling the strength of both inhibitory and excitatory processes in the motor system, its involvement in the integration and transmission of sensorimotor information makes it a contributor to both impairments and possible improvements of neurological motor disorders. Drug therapies aiming to repair the chemical balance between dopamine, glutamate, and other neurotransmitters need to be specific to the disruption at hand so as not to create problematic side effects and further imbalances, and that requires a deeper understanding of the involved systems.

Statement of the Problem

Dopamine has a modulatory influence on motor systems, but this influence varies quite a bit over different circumstances. In the literature cited here, dopamine has been shown to facilitate postsynaptic potentials and induce fictive motor activity in the spinal cord, while it is also involved in motor inhibition. Dopamine exposure can weaken both inhibitory and excitatory chemical influences on motor networks, sometimes at the same time, it can slow and stabilize the rhythmicity of motor activity, and it can disrupt locomotor behaviors. There is still limited knowledge on the patterns and directionality of these effects, which contexts they occur in, and how activity at the cellular level can be connected to activity at the behavioral level. These are things only more research can clarify.

Importance of the Proposed Research

Most studies cited here were conducted on anesthetized animals, or in vitro, and some found modulating effects only on spontaneous activity, not evoked movement. In studying the Mauthner cell, evoked behavior is of greater interest since the escape network is only activated by strong sensory input. Research on dopamine and the Mauthner neuron (Pereda et al., 1992, 1994) looked at the strength of EPSPs, but not at dopamine's effect on overall cell activity or behavior. More *in vivo* studies will be of value in this type of research; recording *in vivo* activity in conscious animals is the most reliable way to examine amine function. The ultimate goal is to understand and repair mechanisms of motor coordination in living creatures, not isolated spinal cords.

Dopamine exposure has been shown to enhance EPSPs in the Mauthner cells of goldfish; I wanted to find out whether it affects overall Mauthner activity levels. This study examined the effect of bath-applied dopamine exposure on the physiological responses of the Mauthner cell to tactile stimulation, in live un-anesthetized zebrafish larvae. The fish were restricted in agar on the recording plate, so actual swimming was not measured, but the presence of slight movements could be recorded, and cell activity was measured as intracellular calcium responses as action potentials fired during attempts at escape movements.

Research Questions and Hypotheses

The goal of this study was to find, in the context of stimulus-evoked activity, a modulated effect on cellular calcium responses and corresponding behavioral responses due to dopamine exposure.

Hypothesis 1. The Mauthner and other reticulospinal neurons will exhibit calcium responses to tap stimuli. Tactile stimulation will startle the larvae and elicit an attempt at movement, illustrated by a rise in calcium activity in the Mauthner cell during action potentials. At the basic level, this experiment will confirm the Mauthner's action in coordinating escape behavior by measuring its activity while evoking a startle response from the fish.

Hypothesis 2. The evoked activity levels will be different in the presence of dopamine than in the control water solution. Previous findings in goldfish and zebrafish have included disrupted spontaneous swimming (Crisp & Mesce, 2004; Xi et al., 2010)

but enhanced Mauthner post-synaptic potentials (Pereda et al., 1992, 1994), implying a facilitation of escape-related swimming. Bath-applied exogenous dopamine exposure is expected to increase overall Mauthner calcium response levels.

Method

Ethics Statement

All animal subjects were raised and handled in accordance with the Humboldt State University IACUC (Approved Protocol # 08/09.P.45.A).

Subjects

Wild-type long fin gold zebrafish (*Danio rerio*) larvae were injected into the spinal cord with a calcium-sensitive fluorescent dye at 4 days post-fertilization (dpf) in order to label Mauthner neurons for recording. Neural recording trials took place at 5 to 7 dpf. Larvae were raised from egg clutches in 10% Hanks solution, a basic saline solution, incubated at 28.5°C on a 12-hour light/dark schedule. All clutches were incubated under uniform conditions from birth. Six subjects were used, in accordance with Gahtan and O'Malley's (2001) criteria for cellular responsiveness and a power analysis.

Instrumentation

Larvae were briefly anesthetized with 0.2% 3-aminobenzoic acid ethyl ester (MS222) and then injected with Oregon Green Dextran (OGD) Bapta-conjugated dye through a glass micropipette beveled back to about a 15μm tip. This fluorescent dye tracker contains a calcium-binding protein which changes structure upon binding to calcium, emitting a temporary higher fluorescence before returning to baseline level after about 2 seconds. The dye increases in brightness in response to the calcium influx and internal release in neurons that are firing action potentials (Figure 2). Cell activity (as

measured by fluorescence intensity) was recorded with an Olympus Fluoview FV1000 confocal microscope and corresponding computer software. Repeated line scans (2ms per scan) captured the fast calcium dynamics associated with action potentials inside neurons. These methods have been used previously and protocols for intracellular calcium imaging in zebrafish are well established (O'Malley, Kao, & Fetcho, 1996).

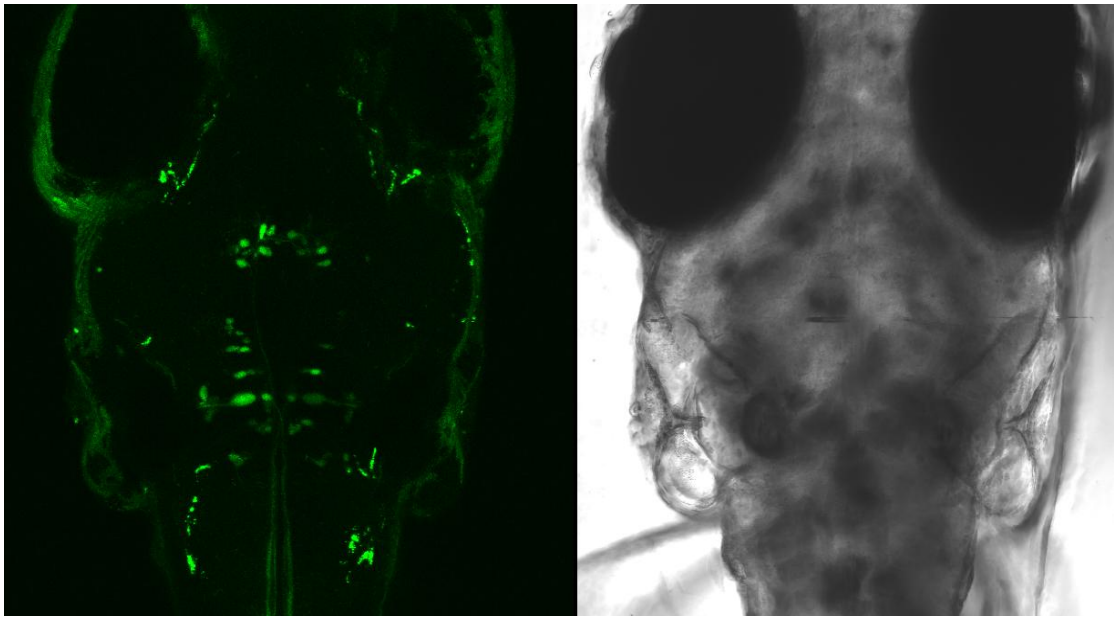


Figure 2. Neurons and tissues of a 6dpf zebrafish larvae fluorescing under laser light after being labeled with OGD Bapta calcium-sensitive dye. Both Mauthner cells are visibly labeled in this subject.

Procedure

Individually anesthetized larvae received OGD Bapta-conjugated dye backfills to retrogradely label hindbrain cells. This was done by pressing a beveled micropipette into the caudal spinal cord and pressure ejecting the fluorescent tracer. After injection, larvae were returned to the incubator in petri dishes to recover for one to two days.

Larvae were embedded in low melting temperature agar during imaging, restricted but not immobilized so as to record real attempts at movement. The recording chamber allowed brain cells to be imaged while the fish was bathed in solutions that could be changed without moving the preparation. The initial condition was a regular bath solution for several trials, followed by a bath solution of 100 μM dopamine hydrochloride, then a regular bath solution again. Repeated line scans of one or both Mauthner cells were imaged in each fish, with two-minute intertrial intervals. Each scan lasted about four seconds, with a total of 2000 lines each. A stimulus was delivered approximately 1 second into the scan, resulting in a period of baseline activity followed by a period of post-stimulus activity scanning. The stimulus was a glass micropipette tapped against the opposite side of the cover slip, known to startle the animal and activate the Mauthner cell. Cell calcium responses are expressed as the percentage change in fluorescence brightness from the pre-stimulus baseline. A fluorescence change over 10% was considered a response, following the criteria established by Gahtan et al. (2002).

Three conditions of trial sets were recorded: pre-dopamine baseline, during dopamine incubation, and after dopamine washout; an ABA experimental design. There were three to five blocks of data in each condition, each block consisting of five trials two minutes apart. The interval between blocks was 20 minutes.

Data Analysis

Cell responses were measured as the average percentage change in fluorescence brightness from the pre-stimulus baseline, in response to stimuli. Repeated-measures

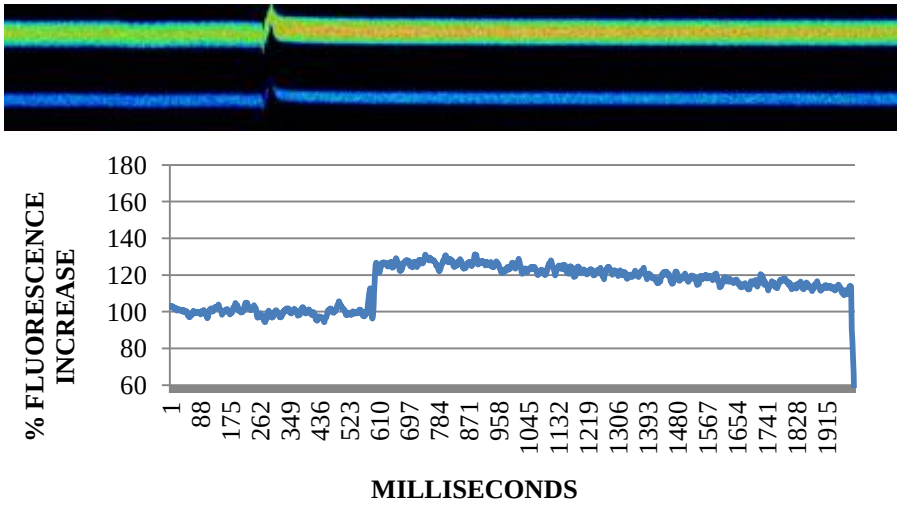
analysis of variance was used to find whether there were significant differences between the baseline condition, the drug condition, and the post-washout condition on four variables: magnitude of calcium response, likelihood of calcium response, likelihood of behavioral response, and response latency. This was done in a group of subjects that received dopamine treatment and in a control group that underwent the same imaging procedures without the drug treatment. Quadratic trends were used to assess curvilinear relationships in the experimental group's data, as I expected a rise and then fall in responses during and after dopamine exposure.

Results

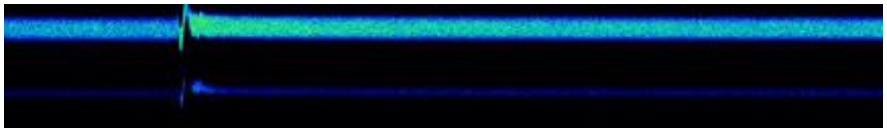
Behavioral Responses to Tap Stimulus

Zebrafish larvae responded behaviorally to the tap stimulus during confocal calcium imaging. Line scans of reticulospinal neurons showed a clear trace of behavior linked to the tap stimulus (Figure 3). This brief (approximately 50ms) "behavioral jitter" demonstrates the effectiveness of the tap stimulus. The break in the line trace sometimes interferes with the ability to measure calcium fluorescence, but usually returns to its previous focal plane immediately. Evidence of physical movement confirms that the escape network is being activated.

Behavioral jitter, which was witnessed in 94% of the trials in the control conditions and 100% of the trials in the dopamine condition, was nearly eliminated after .037% bath application of the voltage-gated sodium channel blocker MS222 (Figure 4), showing water-dissolved drugs effectively penetrate the agar surrounding the larvae and affect the nervous system. MS222 exposure also changed the likelihood and magnitude of cellular calcium responses, but at a slower rate than the behavior.



a.



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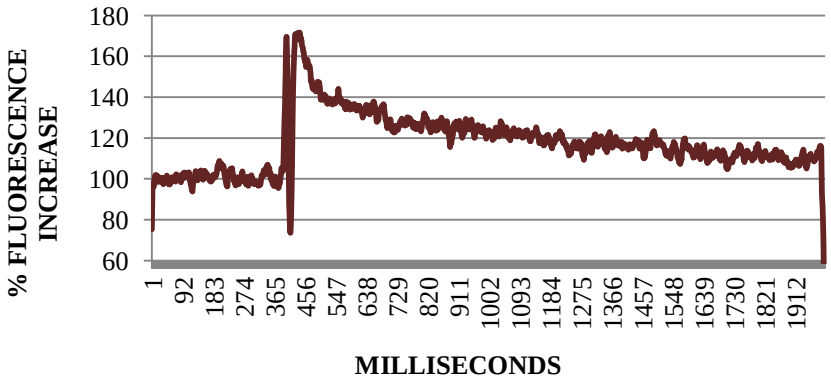

Figure 3. Example double line scans of Mauthner and MiR2, displaying clear behavioral jitters, and corresponding fluorescence intensity traces of the Mauthner cell. *a.* Moderate fluorescence increase of 31.22%. *b.* Large fluorescence increase of 71.65%.

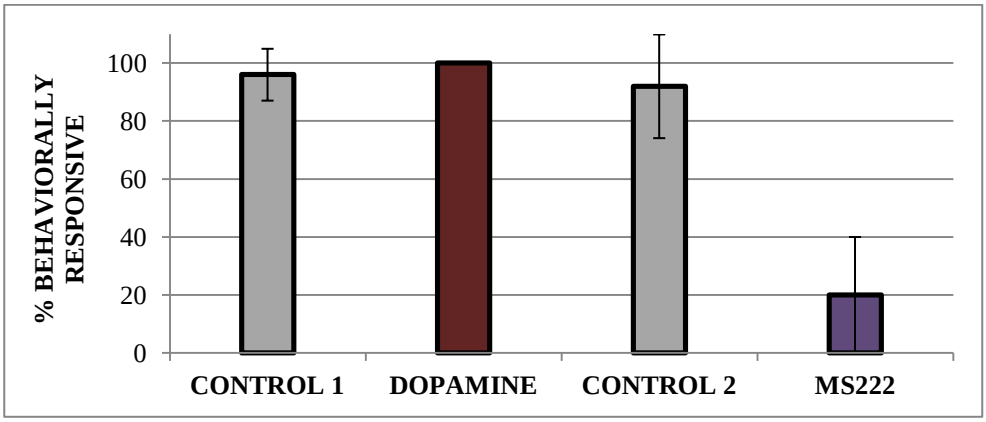

Figure 4. The prevalence of behaviorally responsive trials dropped from 94% to 20% after bath application of MS222. Error bars represent standard deviation.

Mauthner and Other Descending Neurons Show Calcium Responses to Stimulus

Any increase in cellular calcium can be inferred to arise from at least one action potential. A fluorescence increase of 10% was considered a calcium response for a given trial. Previous research has suggested that Mauthner cells only fire one at a time due to inhibitory interneurons between the pair (Gahtan & Baier, 2004), so about 50% responding trials would be expected here since the tap stimulus was non-directional. I found a calcium response in at least 78% of the trials in every experiment.

Dopamine Effects on Tap-Evoked Calcium Responses

Probability of Responding. Dopamine application did not significantly influence the likelihood of a cellular calcium response, $F(1, 3) = 5.69$, $p = .10$, $\eta_p^2 = .66$, but did cause a slight increase in contrast to the control experiment, as tested with a quadratic trend. In the drug-treated subjects, 78% of the trials were responsive in the initial baseline control condition, 92% during dopamine exposure, and 79% in the final

control condition. In the control subjects, 90% of the trials were responsive in the first condition, 97% in the second condition, and 100% in the third condition.

Calcium Response Magnitude. The initial baseline condition had an average post-stimulus fluorescence increase of 20.2% (*SD* = 4.5). The fluorescence increase was higher in the dopamine condition (*M* = 40.2, *SD* = 12.5) and lower in the third control condition (*M* = 30.9, *SD* = 13.8). Quadratic trend analysis showed the magnitude of this calcium response was greatest during dopamine exposure, $F(1, 3) = 19.58$, $p = .02$, $\eta_p^2 = .87$. For the cells recorded in the control experiment, the corresponding second condition (*M* = 51.3, *SD* = 3.1) did not differ from the first (*M* = 49.5, *SD* = 5.8) and third (*M* = 59.7, *SD* = 0.5) conditions, $F(1, 1) = 2.92$, $p = .34$, $\eta_p^2 = .75$ (Figure 5).

Behavioral Responses to Stimulus. In the initial baseline condition, the tap stimulus elicited a behavioral response in 83% of the trials. This rose to 91% upon dopamine application and dropped to 70% after dopamine removal and return to regular water (Figure 6). The difference between conditions, tested with a quadratic trend, $F(1, 3) = 15.03$, $p = .03$, $\eta_p^2 = .83$, was driven by the third condition. Few of the trials immediately following dopamine washout and return to regular water elicited a behavioral response until approximately an hour after the transition.

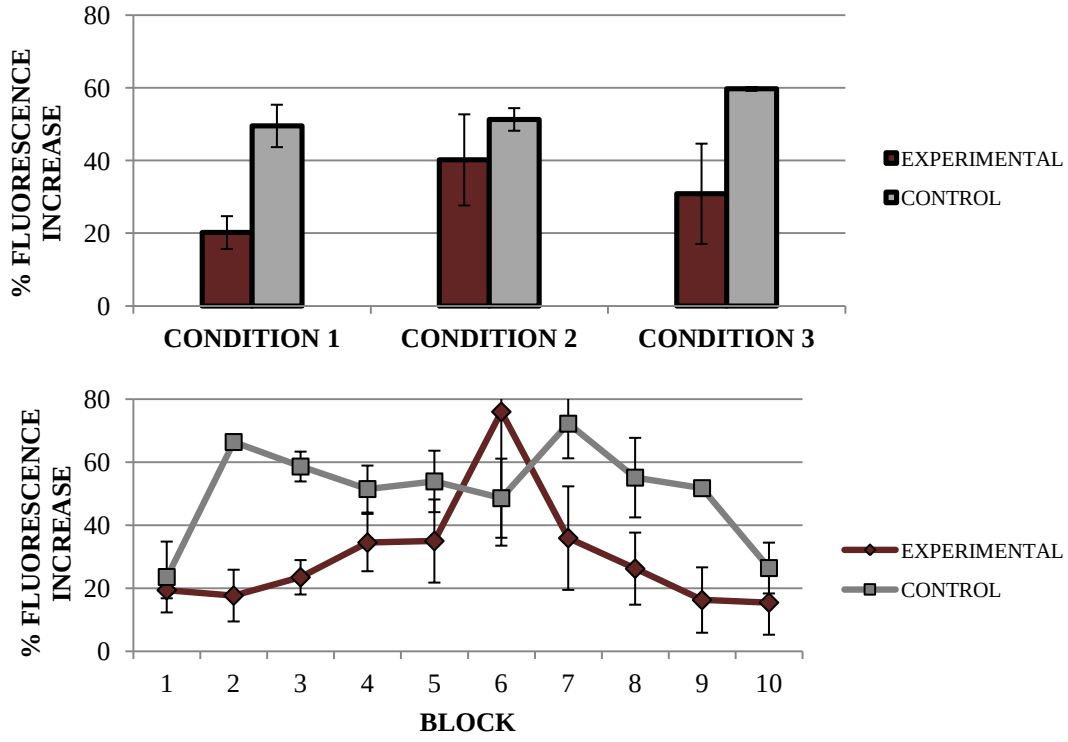

Figure 5. Patterns in fluorescence intensity over time and condition, with larger increases in the experimental group during dopamine exposure. Error bars represent standard deviation.

Response Latency. Response latency between the stimulus time and the point of maximum post-stimulus fluorescence, was extremely variable and not significantly affected by dopamine administration, $F(1, 3) = 6.24$, $p = .09$, $\eta_p^2 = .68$, as tested by a quadratic trend. The average latencies for the drug experiment were 199ms for the first condition, 177ms for the second condition, and 235ms for the third condition; means for the control experiment were similar.

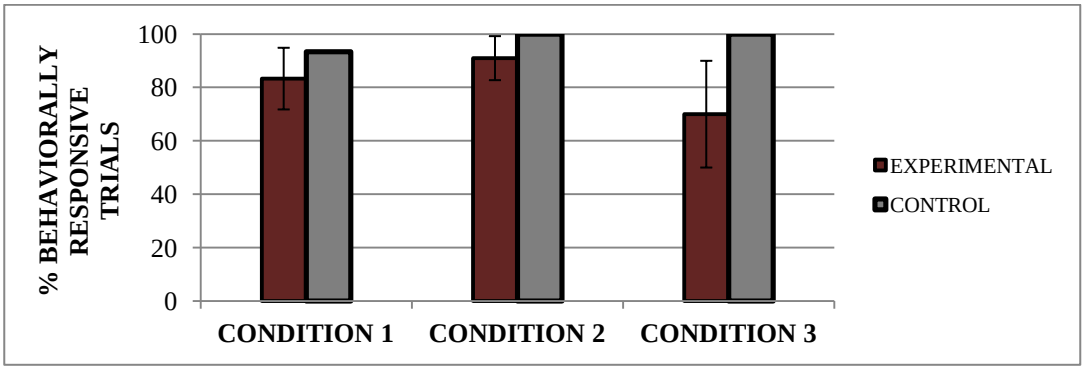

Figure 6. There was a slight increase in the number of behaviorally responsive trials in the dopamine condition, followed by a decrease in the final control condition. This pattern was not witnessed in the control group that was not drug exposed.

Effects on Other Descending Neurons. In two of the subjects, other nearby neurons were labeled coplanar to the Mauthner cells and were also measured and analyzed for dopamine effects. These were identified as MiR2, which projects ipsilaterally down the spinal cord, and MiD2cl, which projects contralaterally. Both have been found to show calcium responses to tap stimuli (Gahtan & Baier, 2004) but it is not explicitly known what function they each have in escape-related movements. The calcium response magnitude of MiD2cl did not change from the baseline control condition (M = 20.5, SD = 7.4) to the dopamine condition (M = 20.6, SD = 4.3), but rose substantially in the final control condition (M = 40.2, SD = 1.9). MiR2, however, had a steep rise in calcium response magnitude between the baseline control condition (M = 19.8, SD = 0.7) and the dopamine condition (M = 97.4, SD = 75.9) due to some high-fluorescence outlier trials, the largest of which was a 492% increase above baseline, before dropping again in the final control condition (M = 46.7, SD = 30.4).

Discussion

All of the larvae were returned to their incubator after the experiments and exhibited healthy blood flow and behavior following the procedures. They did not appear to sustain any damage from the dye injections or drug treatments.

As expected, there was a curvilinear relationship between the condition and the magnitude of the cellular calcium responses, with larger responses during dopamine exposure. There was also a curvilinear relationship between condition and behavioral response likelihood, but this was driven by a steep decrease in movement attempts during the adjustment to regular water after removing the dopamine solution.

Something unexpected was also found, contradicting previous research on the Mauthner array. In one subject, both the Mauthner cells were labeled and positioned coplanar so that they could be scanned and measured bilaterally as a pair. The activity patterns I found in the pair were surprising. As mentioned before, Mauthner cell pairs have inhibitory interneurons between them to ensure that the fish turns away from a tactile stimulus rather than toward it, and are thus expected to only fire one at a time. My findings did not support this expectation. The pair responded together in 66% of the trials in the first condition, 72% of the second condition, and 44% of the third condition. Furthermore, in the first two data blocks of the third condition, following dopamine washout and return to regular bath solution, no behavioral jitter was witnessed, despite calcium responses in one of the cells. This lack of physical movement was typical in most subjects following dopamine washout. However, in both of these blocks, the right

Mauthner was responding in all of the trials, while the left Mauthner never responded, and no behavioral movement resulted from this single-cell activity. This brings to question whether the pair might collaborate under certain circumstances, or whether something about the transition from the drug bath to the regular water bath prevents movement regardless of cellular escape network activity.

Limitations

Because only one fish could be experimented on at a time, and I wanted to use as few animal subjects as possible given the invasiveness of some of the procedures like spinal dye injections, I had a small sample size (n = 6); the large effect sizes of the results suggest that with more subjects I might would have found significant differences for more of the measures. In future research on a longer time scale, a larger sample size and multiple drug concentrations would more clearly illustrate the cellular effects of exogenous dopamine. The means presented here were calculated from "normalized" data; the raw fluorescence values always vary quite a bit in this type of experiment, depending on fluctuating levels of baseline activity and the severity of behavioral jitter in the line scan trace, and there isn't really a reliable way to explain this variation.

There are definitely other ways to investigate dopamine modulation on this motor system; I chose this design in order to have methods that were very specific and that I had successful prior experience with in quantifying cellular responses. Because the design is so specific, the results are also situation-specific, and I would not rule out the possibility of stronger dopamine modulation under different experimental conditions.

Conclusions

Bath-applied dopamine enhanced the calcium response magnitudes in the Mauthner cells of 5 to 7 day old zebrafish larvae, and increased the likelihood of a behavioral escape response to the tap stimulus. Likelihood of calcium responses and latency between the stimulus and the maximum observed fluorescence were not significantly affected. Mauthner cell pairs consistently responded together in the subject that was scanned bilaterally, contrary to prior research that found the cells to inhibit each other and fire separately. In some trials, no behavioral movement resulted unless both cells in the pair responded above threshold. If Mauthner cell activity is indeed enhanced by dopamine exposure, then dopamine modulation appears to facilitate escape-related swimming in larvae while inhibiting spontaneous swimming activity.

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