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ELUCIDATING THE MODULATORY ROLE OF DOPAMINE IN THE C. ELEGANS CHEMOSENSORY ASH NEURON

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ELUCIDATING THE MODULATORY ROLE OF DOPAMINE IN THE *C. ELEGANS* CHEMOSENSORY ASH NEURON

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

by

Cory Kunkel

August 2021

ELUCIDATING THE MODULATORY ROLE OF DOPAMINE IN THE *C. ELEGANS* CHEMOSENSORY ASH NEURON

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Approved by:

Michael Chao, Committee Chair, Biology

Angela Horner, Committee Member

Daniel Nickerson, Committee Member

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ABSTRACT

The neurotransmitter dopamine regulates chemosensory avoidance behavior in the model organism *Caenorhabditis elegans*. Avoidance behaviors are mediated by the polymodal ASH nociceptive sensory neurons, and behavioral avoidance of stimuli detected by ASH is less robust when dopamine signaling is impaired. We are investigating the neural response of the ASH neurons to various stimuli; our investigation includes the behavioral and physiological responses from the ASH neurons as dopamine signaling is manipulated to better understand the effects of dopamine on these sensory neurons. We hypothesize that dopamine plays a regulatory role on the ASH neurons, lessening the response of the ASH neuron to noxious environmental stimuli while the animal is in the presence of adequate food resources. Behavioral assays revealed that *cat-2* mutants, which are dopamine deficient, had decreased behavioral response to the bitter tastant quinine compared to wildtype animals. In addition, normal response to quinine was restored to *cat-2* mutant animals in the presence of exogenous dopamine. Using a custom microfluidics device and the transgenically-expressed Ca^{2+} sensor GFP variant G-CaMP, we found that intracellular [Ca^{2+}] changes in ASH neurons mirrors the behavioral responses in wildtype and *cat-2* mutants, and that exogenous dopamine restores grossly normal response to *cat-2* mutants. The *dop-1* and *dop-4* dopamine receptor mutants had similar behavioral responses to quinine as the *cat-2* mutant while being resistant to exogenous dopamine. However, both

the *dop-1* and *dop-4* receptor mutants showed increased Ca^{2+} influx in ASH neurons when compared to wildtype animals. Collectively our data suggests that dopamine plays a regulatory role on the ASH neurons.

ACKNOWLEDGEMENTS

Dr. Michael Chao

CSUSB College of Natural Sciences, Biology department

Dr. Sreekanth "Shrek" Chalasani, (Salk Institute)

Chao Lab students:

Patricia Turturro

Melvin Baidya

Marissa Torres

Ryan Davis

Jeff Kim

Lauren Valasquez

Jolene Jensen

CSUSB RISE/DIDARP programs:

Dr. Cynthia Crawford

Dr. Paul Orwin

NIH Funding

DEDICATION

This thesis is dedicated to the friends and family that have provided much support and listened to hours technical conversations. To my wife, Katrina, without whose support this would not have been possible. Most of all, this is dedicated to my father, Jerry Kunkel, for his inspiration, support, and for providing the spark of my interest in the field of neurobiology.

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CHAPTER ONE:

INTRODUCTION

1.A. The Neurotransmitter Dopamine is a Monoamine with many Biological Functions

Neurotransmitters are small organic molecules that are utilized by neurons to transmit chemical signals between individual or groups of cells. Neurotransmitters can be classified based upon the structure of the transmitter molecule. The monoamine class of neurotransmitters includes dopamine, serotonin, epinephrine, and norepinephrine; dopamine, epinephrine and norepinephrine can further be categorized as catecholamines (Li et al., 2017). The monoamine neurotransmitters are defined by their containing a single amine group. Deficits in monoamine neurotransmitter signaling have been found to be correlated with various ailments and diseases (Di Giovanni et al., 2016).

1.B. Dopamine Plays a Key Role in Several Neurological Diseases in Humans

Dopamine is known to play multiple critical roles within the human nervous system. It is associated with the control of the locomotor actions, emotion, cognition, sleep/wake cycles, and regulation of the neuroendocrine system (Gugliandolo et al.; Korshunov et al., 2017; Oishi and Lazarus, 2017; Stoker et al., 2017; Volkow et al., 2004).

1.B.1. Parkinson's Disease and Dopamine

One important dopaminergic system in humans involves the dopamine synthesizing region of the midbrain, the substantia nigra compacta. The

substantia nigra innervates the region known as the dorsal striatum, and this pathway is collectively called the nigrostriatal pathway. It is associated with motor control, and degeneration of this particular brain region results in Parkinson's disease (Lang and Lozano, 1998; Vallone et al., 2000). The causes of Parkinson’s disease remain unclear, though many possibilities are being researched. Researchers are looking to genetics, harmful neurotoxins, maladaptive biochemistry, and environmental factors as possible culprits leading to Parkinson’s disease symptoms (Triarhou, 2013).

Parkinson’s disease is best characterized by the death of dopaminergic neurons of the midbrain, reducing the amount of dopaminergic projections connecting to the striatum. The decrease in dopamine within the system manifests as a disruption of body movements; these disruptions appear as hypokinesia, a decrease in the amplitude and frequency of voluntary movement, or bradykinesia, a reduction in the overall speed of movement. In addition to the locomotory deficits, Parkinson’s disease patients frequently suffer from sensory neuron malfunctions manifesting as tingling and pain within the limbs (Rodriguez-Oroz et al., 2009). Increased knowledge gained by research into the basic biological functions of dopamine could aid in the future design of treatments for individuals with Parkinson’s disease.

1.B.2. Drug Addiction and the Role of Dopamine in the Mesolimbic Reward Pathway

Drug abuse is a social concern costing the United States of America billions of dollars a year in treatment, prevention, and law enforcement (Harwood et al., 1984; Mark et al., 2001). Animal models have and will continue to be useful tools in the ongoing quest to understand and thus treat the underlying biological processes resulting in drug addiction (Engleman et al., 2016).

Dopamine plays an integral role in drug addiction (Chiara and Imperato, 1988; Volkow et al., 2004, 2009). Dopamine levels tend to be increased during drug addiction. Both amphetamines and cocaine reportedly increase levels of dopamine release in both the striatum and the nucleus accumbens by up to tenfold and opiates are reported to increase levels of synaptic dopamine (Chiara and Imperato, 1988).

1.C. Dopamine is Found in a Wide Range of Organisms

Dopamine is a conserved neurotransmitter and is utilized in a wide range of organisms from relatively simple nematodes to more complex organisms such as mammals. Some species of dinoflagellates utilize dopamine to regulate water column migration in response to sunlight cues (Forward, 1977). Fruits and vegetables utilize dopamine as a powerful antioxidant molecule to keep fruiting bodies from browning (Udenfriend et al., 1959). Dopamine has even been identified in interspecies interactions such as a host defense response to infectious pathogens (Lyte and Ernst, 1993). Some wasps have been known to

inject dopamine into cockroaches in order to control the cockroaches' behavior so that the cockroaches do not respond to injected wasp larvae (Weisel-Eichler et al., 1999). Infectious fungi utilize host produced dopamine in melanin production in cases of meningitis infections (Williamson et al., 1998). Clearly dopamine is a widely utilized molecule across a wide breadth of taxa.

1.C.1. Dopamine Biosynthesis

Dopamine is synthesized within the neurites (both dendritic and axonal) of dopaminergic neurons from the amino acid precursor tyrosine. The rate limiting step in the conversion reaction of tyrosine to dopamine is catalyzed by the enzyme tyrosine hydroxylase. Tyrosine hydroxylase converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which is then converted to dopamine by the enzyme L-amino acid decarboxylase (Al-Baradie, 2002). The presence of the gene coding for this tyrosine hydroxylase enzyme, encoded by the cat-2 gene in C. elegans, has been used to define the presence of dopamine within a species (Sulston et al., 1975; Vallone et al., 2000).

1.C.2. Signaling Through Dopamine Receptors

Most dopamine receptors are members of the seven-transmembrane domain G-protein coupled receptors; other receptors such as ligand gated chloride channels found in invertebrates can also be activated dopamine (Green et al., 1996; Ringstad et al., 2009). To date, researchers have characterized five unique DA receptors (Kerr and Wickens, 2001; Meador-Woodruff et al., 1992) within mammals. Based upon pharmacological and biochemical attributes, they

have been classified into two main groups: the D1 type (D1 and D5) and D2 type (D2, D3, and D4) receptors (Tarazi et al., 2001). Both receptor types contain regions on the C-terminus that contain phosphorylation and palmitoylation sites, which are thought to be involved in agonist-dependent desensitization of receptors (Bates et al., 1991; Journot et al., 1987).

D1 and D2 receptors are frequently antagonistic to each other; this antagonistic nature allows for the control of motor movements dopamine signaling (Jorgensen, 2004). The D1 and D2 like receptors can achieve this antagonistic regulatory relationship via the activation of different specific intracellular targets. D1 receptors activate adenylyl cyclase, increasing levels of cyclic AMP within the cell (Le Crom et al., 2003) which activates protein kinase A (PKA). Within neurons, PKA is known to phosphorylate various proteins including ion channels, and this alters their conductive properties and affects the excitability of neurons. The D2 like receptors on the other hand are able to act antagonistically to the D1 type receptors by negatively regulating cAMP levels via inhibition of adenylyl cyclase (Beaulieu and Gainetdinov, 2011). For instance, activation of D1 receptors leads to increased Ca^{2+} currents within the cell (Missale et al., 1998).

1.D. *C. elegans* is an Ideal Model Organism for the Study of the Neuro-modulatory Activities of Dopamine

Research into the basic biological functions of dopamine and dopamine receptor activity is relevant in the treatment and understanding of the human

conditions associated with dopamine activity. The study of basic function can be carried be carried out in a multitude of organisms (See section 1C.). For this study, we have chosen the model organism *Caenorhabditis elegans* for a variety of reasons.

1.D.1. *C. elegans* is a Lab Model Organism with a Rapid Life Cycle

C. elegans is a small, free-living, soil inhabiting nematode worm that is a well understood and widely used model organism within multiple research subfields of biology, including neurobiology. *C. elegans* are mainly found as hermaphrodites that produce both oocytes and sperm allowing for self-fertilization; this aspect of the species allows for the maintenance of genetically inbred lines (Brenner, 1974). Groups of *C. elegans* animals can be maintained on small Petri dishes filled with agar and a bacterial food source; on these plates, animals will develop through four larval stages (L1-L4) before becoming a sexually reproducing adult. The maturation of *C. elegans* animals is dependent upon the temperature of the environment ranging from three to five days (15-25ºC) (Blaxter, 2011; Byerly et al., 1976). Large numbers of *C. elegans* can be maintained within the laboratory for minimal cost and effort (Engleman et al., 2016; Sulston et al., 1975). One additional significant benefit to the use of C. elegans as a model organism is that the animal is transparent, which allows for easy imaging of internal structures in vivo (White et al., 1986) . As an invertebrate and a nematode, experimental protocols are not subject to IAUAC review processes.

1.D.2. *C. elegans* has been Investigated with a Wealth of Genetic Tools

C. elegans can be genetically manipulated relatively easily by utilizing ethyl methanosulphonate (EMS) as a random mutagen (Brenner, 1974), producing libraries of mutants (White et al., 1986). *C. elegans* can also be manipulated through reverse genetics, such as the addition of extrachromosomal arrays via transgene injection into oocytes; these transgenic arrays can be passed along to the progeny as semi-stable episomes (Mello et al., 1991). Another reverse genetics tool that *C. elegans* can be investigated with is RNA interference (RNAi). Injections of dsRNA into worms can selectively block the activities of genes complimentary to the injected dsRNA (Gönczy et al., 2000; Tabara, 1998). One of the newest tools for *C. elegans* reverse genetics is that of CRISPR (clustered regularly interspaced short palindromic repeats) driven recombination; this new procedure allows for the creation of both knock-ins (addition of gene/function) and knock-outs (loss of function mutations) at precisely engineered locations, allowing for editing of nearly any genomic region of the species (Chen et al., 2013; Friedland et al., 2013; Gaj et al., 2013). Of particular interest are the varieties of isolated mutant strains exhibiting behavioral differences or aberrations when compared to wildtype worms. Mutations in single genes that lead to differences in behavior illustrate the effect of genetics on the behavior. This is particularly interesting as these mutations in single genes can lead to an understanding of the molecular mechanisms of behavior.

1.D.3. *C. elegans* as a Model for Studying Drug Abuse

C. elegans has been used as a model to study the molecular and cellular mechanisms underlying drugs of abuse, including that for alcohol (Wolf and Heberlein, 2003), nicotine (Waggoner et al., 2000), cocaine (Musselman et al., 2012), and methamphetamines (Schreiber and McIntire, 2011). Invertebrate models offer the ability to relatively easily examine molecular frameworks of drug addiction in a cost-effective way. *C. elegans* offers an array of well understood behaviors to use in investigative assays ranging from egg laying to locomotor gaits (Engleman et al., 2016). The *C. elegans* genome is completely sequenced with approximately 19,000 protein coding genes, allowing for the study of basic biological attributes of specific molecules. This study will utilize the *cat-2* mutant strain of C. elegans that is deficient in dopamine biosynthesis (Sanyal et al., 2004) (See section 1.C.1), to aid in the investigation of the function of dopamine. This mutant strain of *C. elegans* is especially useful due to the fact that in other models, such as the in the common lab mouse *Mus musculus*, deficits in dopamine production can be lethal during fetal development and so are of limited use to researchers (Zhou et al., 1995).

1.D.4. *C. elegans* Utilizes Dopamine for Various Functions

Much has been determined about the functions of dopamine within the *C. elegans* nervous system. Dopamine is believed to be released during times that the animal is in the presence of an abundant food source; the increased release of dopamine in turn make the animal more sensitive to aversive stimuli within the

environment. The logic of this premise is such that animals in an environment that is plentiful in food can afford to be more selective in food choice, avoiding noxious substances, in comparison to an animal that is in a food poor environment.

1.E. Dopamine Plays a Regulatory Role Over many Functions and Behaviors

1.E.1. Locomotion and Food Searching

Animals such as nematodes must locomote to survive. Locomotion is a rich area of behavioral research and has offered many insights into the neurobiology of *C. elegans*. Dopamine has been shown to play a role in multiple *C. elegans* locomotory behaviors. *C. elegans* motor circuits do not function in a deterministic process; instead, sensory inputs affect a continual repetitive series of neuromuscular activities. In other words, animals do not “decide” on a given locomotory path but instead have been shown to locomote in response to environmental cues. Behaviors such as this are carried out via a chain of neurons that can start with sensory neurons communicating with command interneurons; these interneurons are able to elicit effects on motor neurons, causing abrupt changes to direction of locomotion including the initiation of a reversal, or the abrupt change of direction of locomotion. The command interneurons retain some control over locomotion in animals which have had the sensory neurons ablated, indicating the power these command interneurons exhibit over neuromuscular activity. Studies of *C. elegans* on graded plates (plates possessing a gradient of a given chemical that a worm may or may not

find attractive) have shown that the sensory neurons themselves are responsible for the "mapping" of chemical stimuli in the animals' environment (Sengupta and Samuel, 2009).

An example of dopamine-driven behavior is the slowing of well-fed animals when placed on a new food source; this basal slowing is enhanced if the animals in question are not well-fed, indicating a likely role of dopamine signaling in the regulation of this behavior. Animals deficient in dopamine did not exhibit basal slowing; the basal slowing behavior was restored to that of wildtype animals upon treatment with exogenous dopamine. Further, laser ablations of dopaminergic neurons revealed that the multiple classes of *C. elegans* dopaminergic neurons redundantly release dopamine during the activity of this pathway. The data gathered in this experiment suggest a role of dopamine in the control of basal slowing (Sawin et al., 2000; Suo et al., 2004).

Dopamine, in tandem with glutamate, is also known to be involved in the regulation of the area-restricted search (ARS) behavior. This behavior is thought to have evolved to keep animals around abundant food sources. ARS represents an increase in turning rate in the presence of food allowing an animal to remain in a food rich immediate environment. ARS patterns will vary with the time since food was last encountered; the longer the amount of time since food was present the less amount of turning behavior. It is thought that dopamine is released in the presence of food to inhibit glutamate signaling, which in turn increases the

amount of turning exhibited to maintain the animals in the location of food sources (Hills et al., 2004; Vidal-Gadea and Pierce-Shimomura, 2012).

1.E.2. Gene Expression

Dopamine has also been found to affect gene regulation in *C. elegans* through interactions with the transcriptional factor cAMP response element binding protein (CREB) via G protein coupled receptors (GPCR); research has shown implications of this regulatory pathway in learning and drug addiction. Dopamine signaling through the D2-like dopamine receptors DOP-2 and DOP-3 has been found to inhibit release of octopamine (i.e. invertebrate norepinephrine) upon exposure to environments containing adequate food resources. This inhibition of octopamine release results in an inhibition of the CREB protein and thus causes an effect on gene expression. Levels of dopamine within the *C. elegans* nervous system track with levels of acetylcholine. Studies have shown that in conditions of higher dopamine signaling resulted in lower acetylcholine signaling. Upon addition of exogenous dopamine there is also an observable decrease in acetylcholine release as well, supporting the idea that dopamine influences acetylcholine release. This responsive change in neurotransmitter release is slow as the changes depend upon the activity of CREB and CREB being a transcription factor works slower than other signaling molecules. This is a prime example of dopamine signaling affecting the signaling of another neurotransmitter and in turn creating a change in gene expression via CREB inactivation (Suo and Ishiura, 2013).

1.F. Dopamine Receptor Activities Vary by Type

1.F.1. The DOP-1 and DOP-3 Receptors Frequently Function Antagonistically in the Same Pathways

The D1-like and D2-like dopamine receptors (See section 1.C.2.) frequently act antagonistically within the *C. elegans* nervous system. DOP-3, a D2-like dopamine receptor, acts to slow the animals' locomotion. DOP-1, a D1-like dopamine receptor, acts directly antagonistically to the DOP-3 $G_{\alpha 0}$ signaling pathway via the activation of the $G_{\alpha q}$ signaling, as well as the activation of PLCβ signaling cascade. Both receptors have been found to be co-expressed within cholinergic motor neurons as well as individually expressed in other neurons; both receptor types appear to be expressed in levels high enough to effect locomotion from within these cholinergic motor neurons (Chase et al., 2004).

DOP-3 acts via $G_{\alpha 0}$ to execute inhibitory signaling of locomotion within ventral cord cholinergic motor neurons. The activation of the $G_{\alpha 0}$ signaling pathway results in the inhibition of acetylcholine release, thereby lessening or silencing the signals from the cholinergic motor neurons (Allen et al., 2011). The activation of the $G_{\alpha q}$ signaling pathway results in the stimulation of acetylcholine release. Thus, the release of dopamine into the *C. elegans* nervous system can result in the animals' ability to fine tune locomotory activities. Other similar studies suggest that this antagonistic/regulatory relationship between the DOP-3/DOP-1, D2-like/D1-like, dopamine receptors is conserved in mammals (Allen et al., 2011; Chase et al., 2004). At the cellular level the system of control over

locomotory function via co-expressed D1-like and D2-like dopamine receptors is conserved in mammals within the medium spiny cholinergic neurons of the striatum region of the central nervous system, although the precise location of these receptors and thus the specific mode of activity during signaling remains unknown in mammals (Allen et al., 2011).

DOP-1 and DOP-3 have also been implicated in *C. elegans* “choice” making between aversive stimuli. This “choice” is defined as the animal’s willingness to cross a barrier of diacetyl or Cu^{2+}. They are thought to antagonistically regulate behavioral choice via their respective targets. For this particular behavioral choice pathway between aversive stimuli, the DOP-1 receptor is thought to function in cholinergic neurons and the DOP-3 receptor in GABAergic neurons (Wang et al., 2014).

C. elegans naturally exhibit an increase in odorant sensitivity when dopamine signaling is stimulated, such as in times of rich food resources are present. While investigating the role of the various dopamine receptors in this sensory pathway (Ezak and Ferkey, 2010), the *dop-3* receptor mutant animals are the only strain to exhibit a significant increase in the amount of time required for response to 100% octanol. The data generated for the *dop-3* mutant animals suggests that the DOP-3 receptor is required for this enhanced sensitivity to 100% octanol. This activity has been exhibited both in vivo in the presence of adequate food resources and in the presence of artificially increased exogenous dopamine. The role of endogenous dopamine signaling in this pathway is

supported by the phenotypic characteristics of the dopamine deficient *cat-2* animals; the *cat-2* animals do not exhibit the enhanced sensitivity to the octanol stimulus in comparison to the wildtype control animals. DOP-3 expression as assessed by transcriptional GFP reporter transgenes was not observed in either of the sensory neurons ASH, ASI, or in any of the command interneurons. However, transgenic expression of the DOP-3 receptor within the ASH neurons was sufficient to modulate the animals' response to aversive stimuli such as octanol. These findings, in tandem with that of the *cat-2* and *dop-3* mutant animals remaining hypersensitive to octanol exposure, suggest that dopamine likely acts to dampen the signals from the chemosensory neurons themselves. Cell specific RNAi knock-downs of the DOP-3 genes were created within the ASH neurons using the ASH specific promoters *osm-10* and *sra-6*; this resulted in hypersensitivity to octanol that matched the behavior of the *dop-3* mutants. Data from this experiment further supports the idea that DOP-3 is naturally expressed in the ASH neurons, and so is in position to modulate the animals' responses to aversive stimuli. The fact that the DOP-3 receptors were not observed to be expressed in the ASH neurons might be explained by the technical limitations of *C. elegans* transgenic reporter genes. Interestingly, the DOP-1 receptor does not play any antagonistic role within this octanol sensation pathway (Ezak and Ferkey, 2010).

1.F.2. The DOP-2 Receptor is Associated with Various Homeostatic Functions

The DOP-2 receptor is known to negatively regulate various behaviors including egg laying, locomotion, defecation and feeding. This had been illustrated using DOP-2 antagonists to block dopamine signaling by DOP-2 receptors (Suo et al., 2004). Researchers have utilized yeast two-hybrid assays to show that the DOP-2 receptor interacts with the $G_{\alpha i}$ subunit, GPA-14 which is unique to *C. elegans*. The results support the idea that GPA-14 subunit in fact, physically associates with the DOP-2 receptor and so likely acts in an inhibitory fashion. Though no known homologs exist in mammals, the characteristics of this GPA-14 subunit have been sufficient to describe the likely activities of this subunit to be similar to the mammalian inhibitory G proteins (Pandey and Harbinder, 2012).

Activated D2-like receptors are known to negatively regulate adenylyl cyclase. D2-like receptors are known to negatively regulate Ca^{2+} currents via interactions with $G_{\alpha i/o}$ subunits. This regulation is thought to in part have a regulatory action on the biosynthesis of dopamine; some D2-like receptors are found to be both pre- and post-synaptic, poised to regulate the activity of tyrosine hydroxylase (See section 1.C.1), which is Ca^{2+} dependent (Suo et al., 2003). D2-like receptors are also known to regulate K^+ currents within the cell via interactions with different G proteins (Jackson and Westlind-Danielsson, 1994). D2-like receptors located on both pre and post synaptic neurons are able to elicit the down regulation of cAMP levels (Suo et al., 2003).

1.F.3. The DOP-4 Receptor is an Additional D1-like Receptor

The DOP-4 receptor is an analog to the DOP-1 receptor as it too is a D1-like dopamine receptor. Interestingly, studies have indicated that the DOP-4 receptor is functionally active in the ASH neuron. Avoidance behavior to Cu^{2+} and glycerol in the *dop-4* mutant strain is diminished in relation to wildtype behavior; studies have shown that genetic rescues using either native promoters or those that are ASH specific are adequate to restore the mutant behavior to that of the wildtype animals (Ezcurra et al., 2011). Studies conducted on the expression patterns of the DOP-4 receptors using GFP reporter fusion assays indicate that the DOP-4 receptors are expressed in many head and sensory neurons, but are not expressed in the ASH neurons (Sugiura et al., 2005). This contradiction in expression patterns and functional patterns of the DOP-4 receptors creates an interesting topic for future research.

1.G. Dopaminergic Circuits within *C. elegans*

Dopamine has been shown to act synaptically and extra-synaptically within the *C. elegans* nervous system. The aforementioned dopamine regulation of cholinergic motor neurons is a prime example of this. These ventral cord cholinergic motor neurons do not share synaptic connectivity with any of the dopaminergic neurons within the *C. elegans* nervous system (Fig. 1). This lack of connectivity indicates that dopamine must act on these neurons extra-synaptically or humorally as a neuro-hormone (Chase et al., 2004). The ASH neurons do not share any direct synaptic connections with dopaminergic

neurons; thus, if dopamine does play a regulatory role on the ASH neurons themselves it might act in an extra-synaptic fashion, or dopamine might affect the activity of the ASH neuron via connections with other neighboring neurons (Ezcurra et al., 2011; Komuniecki et al., 2014).

1.G.1. *C. elegans* Dopaminergic Neurons

The *C. elegans* nervous system contains eight dopaminergic neurons. In the head can be found a pair of ADE neurons, in the nose two pairs of CEP neurons, and in body can be found a pair of PDE neurons (Fig. 1) (Bargmann, 2006; Sulston et al., 1975). *C. elegans* dopaminergic neurons are likely able to sense bacterial food resources by direct mechanosensory perception (Kindt et al., 2007; Sawin et al., 2000). Animals in the presence of food exhibit an increased sensitivity to soluble repellents (See section 1.D.4.C.1.A.). This behavior has been shown to be directly dependent upon the ability of dopaminergic neurons to sense food in the immediate surrounding environment, such as the ASH mediated behavioral response to aqueous Cu^{2+} (Ezcurra et al., 2011). This is an example of dopamine modulated behaviors related to the food resource qualities in the environment.

1.H. *C. elegans* Expresses Multiple Sensory Neurons Dedicated to the Sensation of Environmental Stimuli

The *C. elegans* hermaphroditic (See section 1.D.1) nervous system is comprised of precisely 302 neurons. This relatively small number of individual neurons comprising the nervous system is a benefit to research as the location

and connectivity of each neuron has been largely identified. *C. elegans* neurons can be grouped into a total of 118 classes of neurons; 20 of the 302 neurons can be classified as pharyngeal and the remaining 282 as somatic (Hall and Russell, 1991; Varshney et al., 2011; White et al., 1986).

Environmental nutritional conditions are a key influence on *C. elegans* behavior (Komuniecki et al., 2014). For instance, in times of abundant food supply *C. elegans* are known to be more sensitive in aversive stimuli such as the heavy metal Cu^{2+} (Ezcurra et al., 2011). The N2 wildtype reference strain of *C. elegans* is known to gather in clumps containing many animals when food is limited in the environment, whereas more "social" strains will exhibit this behavior when food is not limited (de Bono and Bargmann, 1998).

C. elegans have 16 pairs of sensory neurons. These neurons are found in pairs and are classified by the following names: ASE, AWC, AWA, AWB, ASH, ASI, ADF, ASG, ASJ, ASK, ADL, URX, AQR, PQR, PHA, and PHB. These sensory neurons synapse on interneurons and undergo integration prior to synapses with command interneurons, which elicit changes in locomotory behavior directly (Bargmann, 2006; Komuniecki et al., 2014).

1.H.1. The Amphid Pore is the Nematode's "Nose"

Many of the *C. elegans* neurons are located in the head, forming groups of sensory neurons called sensilla. Two of these sensilla are relatively large and form what are known as the amphid pores (Fig. 2), located on either lateral side of the animal. Each amphid pore contains the ciliated endings of eight sensory

neurons, one pair of which are the sensory neurons under investigation in this study, the ASH neurons (White et al.,1986). Amphid neurons also contain axonemal cilia, which are exposed to the external environment to aid in sensation. The ASH neurons are known to contain such axonemal cilia (Kaplan and Horvitz, 1993; White et al., 1986). One can imagine the frequently hostile nature of the external environment faced by worms living in soil, such as infection by pathogenic bacteria (Finlay, 1999; Kurz and Ewbank, 2000) or desiccation (Chakrabortee et al., 2007; Erkut et al., 2013). *C. elegans* as a species may have evolved this amphid pore structure to protect the delicate terminal receptors of the sensory neurons.

This unique structure of the amphid pores suggest that the amphid sensory neurons represent the major chemoreceptor neurons of the *C. elegans* nervous system. Laser ablation of these amphid neurons, such as ASE or ASK neurons, result in the inability of the animal to properly sense its native environment (Calhoun et al., 2015; Troemel et al., 1995). There exists a similar structure in the tail of the *C. elegans* worm known as the phasmid, which is also associated with gathering sensory information from the environment (Hall and Russell, 1991; White et al., 1986).

1.H.2. The ASH Neuron is a Polymodal Sensory Neuron that Drives Dopamine Sensitive Behaviors

The bilaterally paired *C. elegans* ASH neurons exhibit diverse capabilities of sensing aversive stimuli in the immediate environment as polymodal sensory

neurons. This includes stimuli such as high osmolarity, nose touch, volatile chemicals, bitter tastants, and feeding behaviors in social settings (Bargmann, 2006; Ezak and Ferkey, 2010). The ASH neurons function as both chemo- and mechanosensory neurons similar to the vertebrate nociceptor neurons that are associated with the perception of pain in the vertebrate system (Kaplan and Horvitz, 1993). Through behavioral analysis, the ASH neurons are known to sense the following noxious stimuli: Cu^{2+}, SDS (detergent), glycerol, quinine, octanol, and possibly others (Ezak and Ferkey, 2010; Hilliard et al., 2005).

1.H.3. ASH Connectivity/Receptors

The ASH neurons are known to express a multitude of GPCRs with a suite of associated G_{α} subunits such as odr-3, gpa-3 (both expressed in large amounts), gpa-1, -11, -13, -14, and -15 (Bargmann, 2006) (Fig. 2). The ASH neurons are also known to have synaptic connections with the command interneurons AVD, AVB, and AVA (White et al., 1986); these synaptic connections allow for the ASH neurons to directly excite the command interneurons, stimulating them via the release of neurotransmitters such as glutamate and neuropeptides. This excitation of the command interneurons leads to a change in locomotion in response to ASH-sensed stimuli (Hart et al., 1995; Mellem et al., 2002).

1.H.4. ASH Neural Modulation

In food rich conditions the ASH neurons show an increased physiological response, as a quantifiable transient increase in intracellular Ca^{2+} levels, to both

Cu^{2+} and glycerol. In addition to an increase in the magnitude of physiological response, the ASH neurons also show an increase in duration of increased intracellular Ca^{2+} levels in comparison to Ca^{2+} levels in food poor conditions (Ezcurra et al., 2011). Some ASH mediated behaviors have been shown to be modulated by neuropeptide signaling in addition to dopamine signaling (Komuniecki et al., 2014). Clearly food availability influences ASH mediated behaviors and so we hypothesize that food availability affects the activity of the ASH neurons themselves, likely via dopamine signaling.

1.I. Ca^{2+} is the Major Ion Responsible for the Depolarization of the Neurons in *C. elegans*

Ca^{2+} is most likely the major ion responsible for the depolarization of neurons within the *C. elegans*, as the *C. elegans* genome lacks gene sequences for voltage gated Na^{+} channels. Ca^{2+} ions are associated with depolarization and K^{+} ions with hyperpolarization of *C. elegans* neurons (Goodman et al., 1998). Typically, upon stimulation of a sensory neuron, the activation of GPCRs in the sensory cilia results in a signaling cascade that activates Ca^{2+} channels such as the OSM-9 TRP channel found within the membranes of ASH neurons allowing for an influx of Ca^{2+} ions (Feng et al., 2006; Kahn-Kirby and Bargmann, 2006; Xiao and Xu, 2009) (Fig. 3). This property allows for the relatively easy quantification of neural activity based upon transient changes in intracellular Ca^{2+} concentration, thanks to the development of Ca^{2+} sensitive reporter molecules.

1.I.1. *C. elegans* Neural Activity can be Measured Non-Invasively Using Ca^{2+}-Sensitive Fluorescent Reporter Proteins

For the investigation of the neural-modulatory activities of dopamine, we will utilize a modified green florescent protein (GFP) reporter known as G-CaMP (Fig. 4A) that changes fluorescence intensity with changing Ca^{2+} levels. G-CaMP consists of a circularly permutated GFP that has been linked together by the Ca^{2+}-binding domain of calmodulin (Nakai et al., 2001). The calmodulin domain has been inserted into the GFP molecule such that the native β-barrel conformation is broken at low concentrations of Ca^{2+} (Fig. 4C); when Ca^{2+} levels increase, such as when *C. elegans* neurons are activated, the calmodulin domain changes conformation and reconstitutes the β-barrel of GFP. Thus, an increase in fluorescence indicates an increase in intracellular Ca^{2+} levels (Fig. 4C). G-CaMP can be selectively expressed in the ASH neurons (Fig. 4B) by using ASH-specific promoters, such as the promoters for the *sra-6* or *osm-10* genes; this approach has been previously used to measure ASH neuronal activity in other contexts (Guo et al., 2009; Hilliard et al., 2005; Tobin et al., 2002).

1.J. Microfluidics Devices Offer a Novel Means of Conducting Neuro-Physiologic Studies on *C. elegans* Animals

Microfluidics is an example of a crossroads in scientific research. Borrowing from the physics of fluid dynamics, biologists have adapted the use of microfluidic apparatuses to investigate a wealth of biological questions. Microfluidics can broadly be defined as an analytical approach that use fluid

flows on a very small (micro) scale (Mukhopadhyay, 2009). Microfluidics has been in use for some time in tissue engineering and single cell experiments. The use of microfluidics is a useful tool that has allowed neuroscientists to observe the activities of variety of in vivo neurons from a range of organisms, to better understand the ways in which neural circuits work. Microfluidics devices allow for a simple means of immobilizing small organisms such as *C. elegans* for in vivo imaging of cellular activities (Mondal et al., 2012).

Many common microfluidics devices are manufactured by casting polydimethylsiloxane (PDMS); the PDMS polymer is transparent, allowing for microcopy applications. The PDMS polymer is also biologically inert providing a non-interactive microenvironment, and PDMS is relatively inexpensive allowing for the rapid production of large numbers of microfluidic devices in a short amount of time (Mondal et al., 2012; Schrott et al., 2009).

In the past, elaborate protocols for the immobilization of worms had been developed utilizing organic glues such as cyanoacrylic glue to attach worms to microscope slides; though effective, these procedures are technically challenging and require significant time investments per animal immobilized (Richmond, 2009). Another technique used over the years is patch clamp electro-physiologic recordings, which is the gold standard for neurobiologists and in many respects remains the standard. Yet, similar to the organic glue procedures, patch clamp recordings require the difficult dissection of animals ~1mm in length and results in the death of the animals being recorded (Goodman et al., 2012). The

techniques for patch clamp recordings are challenging to master. Modern research techniques are paving the way for the elimination of many difficult to control variables that are associated with invasive means of measuring neuron activity. Researchers have adapted microfluidics systems for the immobilization and study of *C. elegans* animals. The recording chips developed for nematode neurobiology allow for the immobilization of single worms, rapid and precise chemical stimulation, and observations of behaviors in tandem with the precise neural activities that drive them (Chronis et al., 2007). The use of microfluidics devices allows for the immobilization and recording of animals quickly and non-invasively in a short amount of time.

CHAPTER TWO:

MATERIALS AND METHODS

2.A. Animal Maintenance

C. elegans animals were grown using standard methods on 60 mm Petri dishes containing general purpose nematode growth media (NGM) (agar, peptone, cholesterol, 1M $CaCl_2$, 1M $MgSO_4$, 1M KPO_4 buffer at pH=6.0). NGM is applied to plates via a peristaltic pump at 10 mL/plate. Food is provided to the animals via OP50 *E. coli* bacterial lawns, seeded onto the plates at 100 µL/plate. Animals were incubated at a range of temperatures (15-25ºC) over a range of time (3-5 days) respectively (Sulston et al., 1975).

2.A.1 Mating Protocols

Male animals carrying transgenic arrays were created mating male N2 animals with array carrying hermaphrodites resulting in male animals that also carried the array. These male array carrying animals were then mated to homozygous dopamine receptor mutants, representing the parental generation of this cross. F1 heterozygous progeny were selected for via the expression of the G-CaMP array as any animals expressing the G-CaMP were heterozygous for the given mutant gene; selected F1 individuals were singled and allowed to self-fertilize to create F2 progeny. F2 individuals were genetically screened for the presence of the given mutated gene using PCR (see below).

2.B. *C. elegans* Genotyping Protocols

Worm lysates were obtained from individual animals or from entire plates of worms. Plates of worms can be washed from the agar plates using worm lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin), pelleted in a microcentrifuge and aliquoted into PCR tubes. PCR tubes containing live animals were placed in a -80ºC freezer for ~10 minutes prior to the addition of proteinase K (10mg/mL stock solution). Complete reactions were then placed into a benchtop thermocycler, incubated at 60ºC for 60 minutes, and finished at 95ºC for a period of 30 minutes to terminate all enzymatic activity.

Each strain under investigation had DNA primers designed to aid in genotyping each mutant allele. The specific primers can be found in Table 1. The dopamine receptor mutant strains each carry a deletion mutation within the receptor gene allowing for relatively straightforward genotyping. The PCR reactions were as follows: 23 µL H_2O, 3 µL 10X Taq buffer, 0.4 µL 10 mM dNTP mix, 1 µL left primer, 0.5 µL right primer, 0.5 µL internal primer, 0.3 µL 25 mM $MgCl_2$, 0.3 µL Taq polymerase, and 1 µL worm lysate mix (sample DNA) for a 30 µL total reaction volume. Thermocycler conditions are as follows initial denaturation at 94°C for 2 minutes, 35 cycles of the following sequence: 94°C (30 seconds), 55°C (30 seconds), 72°C (30 seconds), and a finishing polish at 72°C for 10 minutes). Genotyping PCR products were identified via gel electrophoresis. Standard reactions were run on a 1.5% agarose gel at 90 volts

for ~90 minutes in the presence of 1X TAE buffer (1 mM EDTA, 40 mM Tris and 20 mM Glacial acetic acid). Completed gels were viewed under a UV light source.

The mutation carried by the *cat-2* animals is a single point mutation requiring the use of restriction fragment length polymorphism (RFLP) to complete a genotyping experiment. The mutation in the *cat-2* (e1112) allele creates an extra cleavage site for the *Dde*I restriction enzyme. This results in an extra *Dde*I fragment in the *cat-2* samples that is not present in the wildtype samples and therefore a different RFLP pattern; we used the larger wildtype (651 base pair) and *cat-2* (616 base pair) fragments to identify *cat-2* genotype. Restriction enzyme reactions were designed as follows: 5.5 µL H_2O, 2 µL 10X BSA, 2 µL 10X Buffer 3, 0.5 µL *Dde*I, and 10 µL of sample DNA as suggested by the enzyme vendor. The *Dde*I reaction was run at 37°C for a period of 2 hours. Products were run in an electrophoresis apparatus that was kept on ice to reduce the temperature of the reaction. A 3% Metaphor gel was used to resolve closely sized DNA fragments, allowing for the genotyping of *cat-2* animals.

2.C. Behavioral Experiments

Behavior was characterized by utilizing an amended protocol from previously published works (Hilliard et al., 2005). Experiments were carried out on foodless NGM plates made to contain 3% less H_2O than standard NGM recipes to create drier plates that facilitated the absorption of the dopamine solution. All animals used for experimentation were young adults raised on plates

incubated at 20 or 25°C. 10 individuals were placed onto each plate and allowed to acclimate for ~5 minutes. Animals were stimulated with aqueous chemicals delivered via flame drawn micropipette tubes (1 MM, VWR International, TW100F-4). Single drops of stimuli were delivered to the agar near the tail of a forward locomoting animal. Capillary action carries the fluid to the animal's nose and the amphid pore (Fig. 5 and section 1.D.6). The time (latency) between delivery of stimuli and the response of the animal (reversal of direction of locomotion) was recorded. Each animal on each plate was only tested once. Data is analyzed as the fraction of animals responding within 4 seconds over the total number of animals tested.

2.D. Microfluidics Experiments

2.D.1. Strains

The following strains were used for this study: N2 (reference wildtype strain), CX10979 *kyEx2865* [*sra-6::GCaMP3; ofm-1::GFP*] (gift from S. Chalasani, Salk Institute), CB1112 *cat-2* (*e1112*), FG58 *dop-4* (*tm1392*), LX636 *dop-1*(*vs101*). All strains except for CX10979 were obtained from the Caenorhabditis Genetics Center (St. Paul, MN).

2.D.2. Microfluidics "Chip" Production

For our microfluidics experiments we utilized an AutoCAD file of the olfactory chip designed by the Chronis lab (Chronis et al., 2007) (Fig. 6); we received this file with much gratitude from Dr. S. Chalasani of the Salk Institute. From this file we had produced a mask to be spincast into a mold by the

Microfluidics Foundry at Stanford University. All future casting made of this mold were conducted using Sylgard 184 silicone elastomer (Sigma-Aldrich 761036-5EA); Sylgard 184 is a two-part elastomer utilizing a 1:10 ration of hardener to elastomer. For our castings we used a 6.4g:64g mixture that was spread over the mold placed within a 150 mm plastic petri dish. The fresh castings were placed in a vacuum desiccator to remove bubbles then allowed to cure for approximately 48 hours. Cured elastomer castings were removed from the mold, scored using a scalpel and individual “chips” were separated prior to punching hose ports using a Harris uni-core tool (internal diameter of 0.75 mm, Fisher scientific NC0711699). Completed chips were them adhered to microscope cover slips (Sigma Aldrich CLS2975245) using a corona generator (Electro-Technic Products, 12051A-10) for approximately two minutes at a distance of 1 cm. Lastly, chip and glass were pressed together, and a piece of tape is applied to apply pressure as well as protect chip surface until the time of use.

2.D.3. Design of Microfluidics Fluid Delivery System

Our microfluidics system requires the use of 5-50 mm syringes used as fluid reservoirs, each connected to the microfluidics chip via small diameter Tygon tubing (ID 0.02 in, OD 0.06 in), using male Luer lock to 0.25 -inch (length) barb adaptors. The other end of each tubing is connected to the fluid ports on the microfluidics chip itself using a small section of metallic tubing (New England Small Tubing, I.D. 0.017 inch—O.D. 0.025 inch). The first reservoir syringe is filled with M13 buffer (30 mM Tris-HCl pH 7.0, 100 mM NaCl, and 10 mM KCl)

(Hart, 2006) and is used to move animals into the microfluidics chip; a three-way Luer lock valve was added in line prior to port 1 on the recording chip (see Fig 6) with a 5 mL syringe added to the third port of the valve. This valve could be set three ways: (1) continuous between syringe and fluid reservoir, so that the syringe can be loaded with buffer; (2) continuous between the syringe and chip as a means to provide pressure or suction while loading worms; (3) continuous between the chip and the fluid reservoir in recording mode. To load a worm, the syringe was first filled with buffer. The tubing was removed from port 1 of the recording chip, and gentle pressure from the syringe was used to place a drop of liquid on an animal on a foodless NGM plate. Gentle suction was then used to pick worm from plate and into the tubing. The tubing was then reattached onto the recording chip at port 1 and gentle pressure was applied so that the worm could be injected into the worm trap. Pressure from this syringe was also used to eject worms from the recording chip post experiment into the waste fluid efflux via the vacuum port. Another reservoir is also filled with M13 buffer, but prior to reaching the chip the tubing meets a three-way solenoid valve (Warner Instruments, Cat. Number 64-0175(SV1)) that can switch the flow of fluid from one hose to another; this assembly is used as an “on/off” switch for the presentation of stimuli to the animal. This general setup was devised by our lab by a previous student, Trisha Turturro. I adapted her design to deliver our dopamine treatment to animals within the microfluidics chip. Another reservoir is again filled with M13 buffer to be exposed to the animal during rest periods. This

rest phase buffer reservoir is also connected to an additional three-way solenoid valve but attached in the reverse direction of flow as the switching reservoir. To this second solenoid valve is also attached an additional reservoir filled with M13 buffer containing freshly added dopamine to treat animals prior to testing. The final reservoir is filled with the stimulus chemical (e.g., quinine) and has a direct connection to the microfluidics ship with no solenoid interruptions. Fluids were pulled through the microfluidics chip utilizing the house vacuum system attached via the same small diameter tubing used to deliver the fluids to the microfluidics chip.

2.D.4. Microfluidics Chip Experimental Setup

Individual chips were checked for defects prior to being used for an experiment. The protective tape was removed from the microfluidics chip and is used to firmly attach the microfluidics chip slide to the stage of the fluorescence microscope. Next the vacuum tubing is attached to the chip (port 2) and allowed to pull air through the microfluids chip for 15-30 minutes. All tubing coming from the fluid reservoirs were primed by pulling fluid through the tubing using a 5 mL syringe. After the tubing was primed, they were individually attached to the chip according to the patterns shown in figure 7. Prior to starting any recording experiments the flow of fluid is established and switching between fluid streams is robustly tested to ensure proper functioning prior to running any experiment. Once this was completed a small amount of immersion oil is added to the

underside of the microfluidics chip slide in preparation for the 60X oil immersion objective lens used during recording experiments.

Experimental animals were grown on NGM plates incubated at 25ºC. Individual animals were removed from the NGM growth plate (containing food) and transferred to an NGM plate containing no bacterial food and allowed to crawl around for a few minutes to “clean” the animals of bacteria prior to experiments. A single animal was loaded into the chip as described above. The animal is allowed to acclimate to chip conditions for a period of 3 minutes total, the last 60 seconds of which it is exposed to blue fluorescence light as the ASH neuron is known to be sensitive to this wavelength of light; pre-exposure to the fluorescent light is able to halt the ASH neuron’s sensitivity to the light exposure (Hilliard et al., 2005). Experimental recordings were obtained using Nikon NIS software and an Andor (model #: DR-328G-CO2-SIL) camera on a Nikon TU-2000 microscope (600x total magnification) (Fig. 10A). Experimental traces were timed as follows: during the first 20 seconds ($t_0 – t_{20}$) of the recording subject animals were exposed to the M13 buffer solution to determine a baseline of fluorescence; between $t_{20} – t_{50}$ subject animals were exposed to the 50 mM quinine-M13 stimulus solution for 30 sec; and finally, between $t_{50} – t_{120}$ the quinine stream is switched away and animals were again exposed to only the M13 buffer solution. This method was utilized to determine the ASH response to the stimulus in each strain of animals. We used the same general recording procedure for the dopamine treatment experiments with the addition of a

prefusion of 4mM dopamine-M13 solution during the first 2 minutes the animals were in the microfluidics chip.

Experimental recordings were initially compiled using the NIS Elements software using the NIS ND sequence acquisition function; this includes both data collection and initial quantification. After each recording that data self-loads for the time measurement function; prior to making this measurement a region of interest (ROI) is added to focus the measurement on the area immediately surrounding the neuron, eliminating external signal noise not created by the ASH neuron. After running the time measurement function the collected data can be exported to Microsoft Excel for further analysis. Data from Microsoft Excel spreadsheets was then imported into Igor (Wavemetrics) for analysis of each recording trace. The procedure was designed to baseline correct each trace, determine the maximum fluorescence, the integral of the trace, the initial slope of response, and the total time of activity. All data points were then transferred to JMP (SAS) to determine the statistical significance of the results. Physiologic experimental data were analyzed using the Student's t-test and one-way ANOVA with Tukey-Kramer method to compare means; behavioral data were analyzed using Student's t-test; significance was set at $p<0.05$.

CHAPTER THREE:

RESULTS

3.A. Endogenous Dopamine Appears to Play a Role in Control of *C. elegans* Nociceptive Behaviors

To determine the neural-modulatory role of dopamine in the *C. elegans* chemosensory avoidance behavior, we assessed behavioral differences between wildtype and the dopamine deficient *cat-2* mutant strains (See section 1.C.1). We tested a panel of known ASH-sensed soluble stimuli (See section 1.H.2.). When a drop of liquid containing a noxious substance is applied to a forward moving *C. elegans* animal it responds by a rapid reversal of direction. The data for responses were quantified in two ways: (1) a response was scored if the animal reverses direction within four seconds of being stimulated and is reported as a percentage of animals responding and (2) the actual time elapsed was recorded from presentation of stimuli to the beginning of a reversal of direction (Fig 5). In most cases, wildtype animals will respond with a reversal in direction for nearly all animals tested. Dopamine deficient *cat-2* mutant animals were observed to be less responsive to stimulation with glycerol and quinine in comparison to the wildtype animals. Only 50% of *cat-2* animals responded to the glycerol stimuli compared to >75% of wildtype responding. A more robust difference was observed with the quinine stimuli, resulting in <20% of *cat-2* animals responding compared to nearly 100% of wildtype animals responding. *cat-2* animals responded better than wildtype animals when presented with 0.1% Cu2+; >85%

of *cat-2* mutant animals responded more rapidly compared with only ~50% of wildtype animals responding. In summary, we found that for most of the repellents tested, the dopamine deficient *cat-2* animals responded less to aversive stimuli; an interesting result was the increased response of the *cat-2* animals to the heavy metal Cu^{2+} in comparison to the wildtype animals. These results suggest that dopamine signaling may play a role in regulating *C. elegans* response to soluble aversive stimuli, although different stimuli may be regulated different ways (Fig. 8).

To further confirm that the behavioral differences observed between the wildtype animals and the *cat-2* animals could be attributed to a deficiency in dopamine, we asked whether exogenous dopamine could restore normal behavior to *cat-2* mutant animals. We repeated the previous behavioral experiments but tested the animals in the presence of exogenous dopamine. There was no significant effect of the dopamine treatment on wildtype animals. The dopamine treatment yielded no significant difference in the behavior of the wildtype animals. The response of the *cat-2* animals in the presence of exogenous dopamine was restored to the normal response observed with the wildtype animals (Fig. 9); with the addition of dopamine, ~90% of *cat-2* animals responded to quinine. This led us to also evaluate response to quinine in *dop-1* and *dop-4* mutant animals. As expected, both dopamine receptor mutants had poor responses to quinine; ~45% of *dop-1* and ~55% of *dop-4* animals responded to quinine. Exogenous dopamine treatment of the dopamine receptor

mutants had no effect on response (Fig. 9). These results suggest that dopamine is required for regulating quinine sensation. Also, these results show that dopamine can be applied to *C. elegans* exogenously to replace the missing endogenous dopamine, suggesting that dopamine may be functioning extrasynaptically as a neuro-hormone rather than as a traditional synaptic neurotransmitter. This is consistent with previous studies using volatile odors detected by ASH neurons (Baidya et al., 2014).

3.B. Microfluidic Devices in Conjunction with G-CaMP Reporter Proteins Allow for the Quantification of Ca^{2+} flux within *C. elegans* Neurons

To make a quantitative assessment of ASH neural activity in the *C. elegans* animals, we used a transparent microfluidics device (See section 1.J.) to immobilize the animals and to allow for the rapid application and removal of stimuli; the effects of the stimuli were quantified using the G-CaMP calcium reporter protein expressed in ASH neurons (See section 1.I.1.). The microfluidics device is mounted on a fluorescent microscope to enable us to create video recordings of the change in fluorescence signal upon stimulation of the animals. The various aqueous solutions that will be delivered to the animal within the microfluidics device are contained in a series of fluid reservoirs (Fig. 7 and 10). The flow channels in the microfluidics chip are connected to these reservoirs via small diameter tubing, allowing for the delivery of fluids to the animal being tested (See section 2.D.). The microfluidics device is also connected to a vacuum source, which drives fluid flow. The addition of small solenoid valves in-line

between the reservoirs and the microfluidics device allows for the rapid and precise switching of the fluids to which the animal is exposed. Animals at rest are exposed to a saline buffer or possibly an experimental treatment (i.e. exogenous dopamine) and activating a valve exposes the trapped animal to a stimulus. A closer look at the microfluidics device reveals the architecture of the worm trap that immobilizes the animal in such a way that the nose is exposed to the channel carrying the switchable fluid stream (Fig 10C).

To determine the resolution of the G-CaMP reporter proteins in the evaluation of neural physiology we performed a series of experiments with *C. elegans* to develop a procedure to quantify the activity of the ASH neuron. For physiological recording experiments, we have used a strain of worms that expresses the transgenic G-CaMP reporter proteins within the ASH neurons in an otherwise wildtype background using the *sra-6* promoter (*sra-6::G-CaMP*). We measured Ca^{2+} flux as a proxy for the physiological response of ASH neurons to stimulation with 1M glycerol. The fluorescence trace shows the average values of the ASH neurons physiologic activities (Fig. 11A). To normalize the data, we averaged twenty seconds of fluorescent signal while the neuron is at rest to represent a baseline of activity; all the data points for an individual video recording are then normalized by this baseline value to show an increase in fluorescence as a percentage. The normalized data are then averaged over multiple recordings from individual animals ($n > 10$). During the first twenty seconds of each recording the animals are exposed to saline solution only; next

the animals are exposed to the stimulus for a period of ten seconds, and lastly the animals are exposed to saline again (Fig. 11) this represents an entire data collecting recording. In later experiments we adapted this procedure to allow for a longer, 30 second, exposure to stimulus to further investigate the kinetics of the ASH neuron's activity.

Is there an observable difference in the physiological activity of the ASH neuron associated with the level of endogenous dopamine within an individual animal? By utilizing a strain carrying the transgene in a *cat-2* background (*cat-2; sra-6::G-CaMP*), we can further understand the effects of dopamine on the ASH mediated behaviors (Fig. 12). The dopamine deficient *cat-2* mutant strain is known to produce ~40% less dopamine compared to wildtype animals (Sanyal et al., 2004), The *sra-6::G-CaMP* animals responded to the glycerol with a ~150% increase in normalized fluorescence intensity over the baseline (Fig 12A). *cat-2 sra-6::G-CaMP* animals responded to the glycerol stimulus with a ~145% increase in normalized fluorescence signal, which was not significantly different from the way that the *sra-6::G-CaMP* responded (Fig. 12B). This line of experimentation failed to yield data indicating a significant difference in the peak fluorescence, as determined by students t-test, in the ASH neuron response as measured as Ca^{2+} flux in the presence or absence of endogenous dopamine. However, the data does suggest that we have developed a means to quantify the activity of the ASH neurons; our quantified ASH activity traces resemble those of multiple published works (Chronis et al., 2007; Hilliard et al., 2005).

3.C. Dopamine Activity is Diminished in the Presence of Salt

C. elegans exhibits a thrashing behavior when placed in water. Interestingly the addition of dopamine to water will paralyze the animals eliminating the thrashing behavior. This assay is known as swimming induced paralysis (SWIP) (Hardaway et al., 2012; McDonald et al., 2007). We have utilized this behavior to determine the activity of dopamine when suspended in the isotonic saline buffer solution M13 (see Materials and Methods), as M13 is used as the standard recording buffer and will therefore be used to deliver dopamine to animals trapped within the microfluidics device in our proposed experiments. We found that the biological ability of dopamine to render *C. elegans* animals paralyzed is diminished when the dopamine is suspended in M13. 4 mM dopamine dissolved in polished H_2O can paralyze >50% of animals within 3 minutes of application. The paralyzing effects of dopamine was diminished when the dopamine was dissolved in M13. When the dopamine is dissolved in the M13 the concentration of dopamine must be increased to 300 mM to induce the same level of paralysis as 4 mM dopamine in water (Fig. 13A). We assessed what solutes within the M13 recording buffer are responsible for the diminished activity of dopamine in this assay by individually testing each component of the M13 buffer with dopamine and assessing paralysis. Our data indicates that both KCl and NaCl have an adverse effect of the ability of dopamine to render the animals paralyzed; in the presence of either salt nearly zero animals were paralyzed. The Tris buffer had no effect on the activity of

dopamine; nearly 100% of animals treated with dopamine and Tris buffer were rendered paralyzed (Fig. 13B). The data generated from the SWIP assays have given us a working concentration of dopamine (300 mM) to match the activity of 4 mM dopamine in water..

3.D. ASH Neuron Response to Quinine

Although we did not find any difference in ASH Ca^{2+} response between wildtype and *cat-2* mutants when using glycerol, we decided to test if we could detect a difference using quinine instead, as behavioral response to quinine in the drop assay exhibited the most robust difference between wildtype and *cat-2* (Fig. 8) (See section 3.A.). We initially used 10 mM quinine as stimulus for Ca^{2+} recordings because this concentration provided robust responses in behavioral experiments. However, stimulation with 10 mM quinine did not yield any detectable response. To remedy this problem, we attempted experiments with increasing concentrations of quinine. 50 mM quinine yielded results that we were able to quantify and so this became our stimulus concentration for all future recording experiments.

We measured transient changes in intracellular [Ca^{2+}] in *C. elegans* ASH sensory neurons in response to chemical stimuli quinine (See section 3.A.) For each experimental condition we obtained recordings from 20 individuals. We initially analyzed the recording traces to establish the intensity of response; after many iterations of the experiment, it became clear that certain individuals simply did not respond. One explanation for this is that animals not producing a

response might be damaged/injured in the microfluidics chip loading process (but see also Discussion). We chose to exclude these responses from our statistical analysis, but we report fractions of animals responding. We set a threshold of >20% increase in peak fluorescent signal to include a trace in the statistical analysis. We then analyzed the traces to determine the following metrics (Fig. 14). The peak fluorescence of response was measured as the maximum percentage increase in fluorescence signal over the established baseline resting level. The total time of response was measured as the amount of time that the fluorescence signal was greater than that of the pre-stimulus baseline. The integral of the traces was measured as the area under the trace between application of stimulus to when fluorescence level returns to pre-stimulus baseline. The slope of the initial increase in fluorescence signal is measured as the initial slope of response. We also measured the time elapsed from stimulus initiation to the peak fluorescence as the time to peak. Prior to analysis the traces were normalized to zero using the pre-stimulus rest period data points. These metrics were used to evaluate the activity profiles of the ASH neurons.

We found that ASH neurons in wildtype animals (n=17/20) responded with a significantly greater peak of fluorescence (111.3% ± 13.2 $\Delta f/f$) in comparison to *cat-2* animals (n=7/20) (56.8% ± 16.6 $\Delta f/f$) and the integrals of response (34914.8 ± 5572.6 ($\Delta f/f$)·(seconds)) and (16339.4 ± 6985.6 ($\Delta f/f$)·(seconds)) respectively, as expected based on initial behavioral experiments. There was no statistical difference between the ASH neurons total time of response, slope or

time to peak fluorescence between wildtype (Fig. 15A and 16) and *cat-2* (Fig. 15B and 16). Interestingly, the fraction of ASH neurons in wildtype and *cat-2* animals responding to quinine in this experiment resembled the fraction of animals of their respective genotypes that respond behaviorally (see section 3.A.). The data suggests that endogenous dopamine signaling affects the overall magnitude of Ca^{2+} flux in ASH neurons in response to quinine without changing the overall kinetics.

3.E. Exogenous Dopamine Restores Normal ASH Response of *cat-2* Animals

We next tested the ASH response of dopamine treated animals for comparison with non-treated animals. SWIP assay data (see section 3.C.) indicated the need to greatly increase the concentration of dopamine to maintain physiologic functions in the M13 buffer solution. However, early experiments using the 300 mM dopamine-M13 solution gave rise to technical problems. Usually in a typical recording experiment, we are able to process several animals allowing for the acquisition of several traces using a single microfluidics chip. Individual animals are injected into the microfluidics chip, tested, and then expelled using syringe to push the worm out of the trap and eventually into the vacuum (port 2); a second worm can be then loaded and the process repeated. Using 300 mM dopamine we were only able to test a single animal per microfluidics chip, as each animal became stuck just outside of the trap never arriving at the vacuum port. We believe the excess dopamine created a more viscous solution not allowing for the ejection of the tested animal. We also

viewed this dramatically increased concentration of dopamine as unrelatable to the significantly lower concentrations of 4 mM used in behavioral assays. To remedy problems of viscosity and of potential unrealistic dopamine concentrations, we decided to attempt treatment with the 4 mM concentration of dopamine-M13 solution, even though 4 mM M13 showed little activity in the SWIP assay. We knew from the previous SWIP assays that the stability of the dopamine in the M13 buffer declined over time, but would it remain stable enough over the short term to allow for treatment of animals? To ensure that each dopamine treatment had equivalent effects we created the 4 mM dopamine M13 solution immediately prior to each experimental trail and completely flushed the previous treatment mixture from the system prior to proceeding with the next trial. This was a time-consuming process but much more efficient that the experimental trials with the 300 mM dopamine M13 solutions. Exogenous dopamine was perfused within the microfluidics recording rig for a period of 60 seconds prior to running the before mentioned experimental recording procedure. As seen in figures 15B and 16A, 4 mM dopamine treated *cat-2* animal ASH neurons (n=9/20) responded with a significantly greater peak fluorescence (263.0% ± 55.5 $\Delta f/f$) when compared to non-treated *cat-2* animals (Fig. 15D and 16A) ASH responses (n=7/20) (56.8% ± 16.6 $\Delta f/f$). We also observed a significant difference in the integral of the ASH response traces when comparing the dopamine treated *cat-2* animals (79868.8 ± 17891.6 ($\Delta f/f$)·(seconds)) with the non-treated *cat-2* animals (16339.4 ± 20287.1 ($\Delta f/f$)·(seconds)): a ~480%

increase (Fig. 16C). We observed no significant difference in the comparison of the following metrics: total time of response, slope, or time to peak fluorescence as seen in figures 16. We continued with the 4 mM dopamine treatment of wildtype animals. Comparing dopamine treated (n=13/20) (193.9% ± 39.49 Δ*f/f*) versus non-treated (17/20) (104.2% ± 41.1 Δ*f/f*) wildtype animals (Fig 15A,C and 16A), ASH responses to 50 mM quinine revealed a wider variance in response but not a statistically significant difference in the means. Again, we observed no significant difference in any of our comparative metrics as seen in figure 16. This data suggests that exogenous dopamine treatment partially restores normal ASH activity in dopamine deficient *cat-2* animals.

We next compared 4 mM exogenous dopamine treated wildtype and *cat-2* animals. We observed no significant differences in the peak fluorescence, total time of response, the integral, slope or time to peak between wildtype and *cat-2* ASH neurons as seen in figure 16. Interestingly, the OFF response in dopamine-treated *cat-2* animals was greatly increased. These data further suggest that endogenous dopamine influences ASH neuron activity in response to quinine, although the exogenous dopamine treatment of dopamine deficient *cat-2* mutants does not fully recapitulate the wildtype response.

3.F. ASH Neurons in DOP Receptor Mutants Respond to Quinine more Robustly than Wildtype Animals

With good evidence supporting the hypothesis that dopamine plays a regulatory role on the ASH neuron activity we further desired to understand what

dopamine receptors may be at play in this regulatory pathway. As previously discussed, good candidate dopamine receptors for investigation are *dop-1* and *dop-4* (See section 1.F.). We recorded ASH Ca^{2+} response traces to 50 mM quinine for both *dop-1* and *dop-4* mutant strains of C. elegans animals. We first compared the ASH response curves between *dop-4* mutant animals (Fig. 15F) with that of wildtype animals (Fig. 15A). The ASH response trace of *dop-4* animals (n=17/20) showed no significant differences in the metrics of peak flouirescence, slope, integral of response, total time of response or the time to peak fluorescence when compared to wildtype animals as seen in figure 16. We also observed significant differences in peak fluorescence between *dop-1* animals (Fig. 15E) (n=19/20) (556.1% ± 69.5 $\Delta f/f$) and wildtype animals (Fig. 15A) (n=17/20) (104.1% ± 87.5 $\Delta f/f$) (Fig. 16A). This difference was massive - a greater than a 4-fold increase.. Next, we observed a significant difference in the integral of the curves between *dop-1* animals (168447 ± 29128.9 ($\Delta f/f$)·(seconds)) and wildtype animals (34915 ± 38282.9 ($\Delta f/f$)·(seconds)) (Fig. 16C); the *dop-1* ASH response had a ~480% increase in the integral compared to wildtype animals. Additionally, we observed a significant difference in the initial slopes of the curves comparing *dop-1* animals (44.1 ($\Delta f/f$)(sec.) ± 10.0) and wildtype animals (9.1 ($\Delta f/f$)(sec.) ± 12.6) (Fig. D). Lastly, we observed a significant difference in the time to peak fluorescence between *dop-1* (24.7 ± 2.3 seconds) animals and wildtype (24.5 ± 2.9 seconds) animals as seen in figure 16. Finally we compared the ASH response profiles to 50 mM quinine between

dop-1 (Fig. 15E) (n=19/20) and *dop-4* (Fig. 16F) (n=17/20) mutant animals. We observed a significant difference while comparing the peak fluorescence of *dop-1* animals (556.1% ± 72.0 Δ*f/f*) and *dop-4* animals (314.5% ± 79.1 Δ*f/f*) (Fig. 16A). We found no additional significant difference in any of the remaining metrics of total time of response, the integral of the curves, the initial slopes of the curves, or the time to peak fluorescence comparing *dop-1* animals to *dop-4* animals as seen in figure 16. Contrary to our initial expectations these data suggest the DOP receptor mutants have a more robust Ca^{2+} response in ASH neurons than wildtype animals, discussed further below.

CHAPTER FOUR:

DISCUSSION

The experiments described above in Chapter 3 strongly suggest that dopamine influences the response of the ASH neurons to the noxious stimuli quinine. Behavioral experiments revealed significant differences in the behavior of *C. elegans* animals with different levels of endogenous dopamine; treatment of dopamine deficient *cat-2* mutants with exogenous dopamine restored behaviors to resemble those of normal animals (Fig. 7). ASH neurons are known to detect noxious environmental stimuli such as quinine (Bargmann, 2006; Ezak and Ferkey, 2010), yet the question remains as whether dopamine influences the activity of the ASH neurons causing the behavioral differences observed. In physiological experiments utilizing the G-CaMP fluorescent reporter to provide a Ca^{2+} flux trace as a proxy for neural activity, we were able to demonstrate that treatment with exogenous dopamine restored the Ca^{2+} activity of the ASH neurons in *cat-2* dopamine deficient animals to that of wildtype animals (Fig. 15C and 16).

Early behavioral experiments within our lab revealed that M13 recording buffer substantially reduced the activity of dopamine. SWIP assay data (see section 3.C) suggested that the ability of dopamine to render the animals paralyzed was greatly diminished at standard 4 mM concentrations in the presence of the M13 recording solution. To get around this problem, we asked whether greater concentrations of dopamine in the M13 buffer could elicit the

same effects observed with lower concentrations of dopamine in H_2O; experiments revealed that 300 mM dopamine in M13 had the same paralytic effects as 4 mM dopamine in water. With this new “working” combination of dopamine and M13 buffer we attempted to treat animals within the microfluidics chip, but this approach proved to be problematic. Experimental throughput was severely impacted as only one animal was able to be processed through each microfluidic chip after which the chip became unusable, requiring a time-consuming switching and preparation of microfluidics chips in between trials. Something about this high concentration of dopamine was causing animals to become stuck within the microfluidics chip; we hypothesized that the solution had become more viscous or sticky causing the animals to become stuck. We also hypothesized that the paralytic effect of the dopamine may play a role in preventing the animals from being ejected from the microfluidics chip.

This left us in a predicament; we desired to treat the animals with dopamine inside of the microfluidics chip, but to maintain separation of fluid flows within the microfluidics chip as well as to maintain similar osmotic concentrations, the dopamine had to be delivered in the M13 solution. The SWIP assay presumably targets ventral cord motor neurons that are known to express dopamine receptors (Hardaway et al., 2012; McDonald et al., 2007). Previous experiments showed that lower concentrations of dopamine in M13 could influence ASH driven behavior (see section 3.A.) (Ezak and Ferkey, 2010), which are done on NGM plates that contain similar salt concentrations as M13 buffer.

The ASH neurons are also amphid neurons (see section 1.D.6.) with sensilla that are directly exposed to the microfluidic buffer stream, as opposed to the ventral cord motor neurons that are located more posteriorly and therefore "shielded" by the PDMS chip. If dopamine receptors are expressed in ASH, it is possible they would be more accessible to dopamine in the buffer stream even at lower effective concentrations. Given these considerations, we attempted to use lower concentrations of dopamine in M13 in our recording chip to see if we could observe any effects. This approach allowed for successful exogenous dopamine treatments in the microfluidics chip, providing data that clearly and significantly illustrated the effect of dopamine on the ASH neurons (see section 3.E).

We observed some differences between our ASH Ca^{2+} recordings and similar previously published data. As discussed in section 1.H. the ASH neurons exhibit a peak of activity at the onset (the ON response) of stimulus that rapidly decays but does not return to baseline; the neuron shows a steady and elevated level of Ca^{2+} until the stimulus is removed. The removal of the noxious stimuli triggers an OFF response, which is characterized by another spike in intracellular Ca^{2+} followed by a rapid drop-off back to baseline (Chronis et al., 2007; Hilliard et al., 2005). This ON/OFF response was characterized using glycerol (i.e., hyperosmotic shock) as a stimulus for ASH neurons. ASH neurons were also shown to have a Ca^{2+} response to quinine (Hilliard et al., 2005), although an ON/OFF response was not observed in that study. Overall, the recordings we obtained using quinine were grossly similar to published results. Our ASH traces

of both wildtype and *cat-2* animals had the marked ON and OFF response peaks to application and removal of quinine but the ON response was more persistent and did not decay as quickly. The amplitude of our Ca^{2+} peaks (~80% increase) were also lower than reported values for ASH neurons (~180% increase).

There might be several explanations for these discrepancies. In Hillard et al (2005) a different Ca^{2+} sensitive reporter protein cameleon was used to measure quinine responses. Cameleon uses a different mechanism for Ca^{2+} induced fluorescence than G-CaMP (Looger and Griesbeck, 2012). Furthermore, cameleon reporters are sensitive to motion artifacts, and worms are physically glued to an agar pad to facilitate recording; this may have affected ASH neurons in some way that obscured an ON/OFF response. We adapted the microfluidics chip approach that Chronis et al (2007) used and saw a similar ON/OFF response for quinine as they did for glycerol, suggesting that an ON/OFF response is a general feature of ASH neurons. The differences in the decay of the ON response might be attributable simply to differences in stimuli (glycerol vs quinine). Finally, although we used (from a behavioral standpoint) a very strong stimulus of 50 mM quinine, the amplitude of the Ca^{2+} spike was relatively weaker than previously reported for ASH neurons. We note that our preliminary experiments with glycerol were also substantially weaker than published results (Fig. 12) and we did not observe ON/OFF responses with glycerol treatment even using the microfluidics chip. It is possible that local environmental conditions in our lab simply yield weaker ASH responses in general.

Our data suggests that treatment of dopamine deficient *cat-2* with exogenous dopamine can increase the overall activity of the ASH neuron in response to the noxious stimulus quinine; this increase in activity grossly resembles that of the ASH activity of exogenous dopamine treated wildtype animals (Fig. 15). Similar to published works we saw a near doubling in the amplitude of exogenous dopamine treated wildtype animals in comparison to non-treated animals (Ezcurra et al., 2011) (Fig. 15). That exogenous dopamine can increase Ca^{2+} activity in wild type animals is not surprising, given that it takes at least 5 minutes to load an animal into the microfluidics chip, during which it is not exposed to food. *cat-2* ASH activity was increased to a similar peak and overall general activity as wildtype; this represents a nearly 400% increase in comparison to non-treated *cat-2* ASH activity. In addition, ASH activity observed in dopamine treated *cat-2* animals had grossly exaggerated OFF responses when compared to the ON response. This was evident when comparing our wildtype results or with other published data (Chronis et al., 2007; Hilliard et al., 2005). Even though we do not see the same OFF response increase in wildtype animals, we also do not see the same percentage increase in overall ASH activity; this indicates that the exogenous dopamine treatment had a stronger effect on ASH activity in *cat-2* mutant animals. It is therefore reasonable to conclude that exogenous dopamine treatment largely restores normal ASH neuron Ca^{2+} response to quinine in *cat-2* mutants, and that the differences

between *cat-2* and wild type ASH neuron Ca^{2+} responses can be attributed to a dopamine deficiency.

Interestingly, when we tallied the percentage of animals responding with increased ASH Ca^{2+} activity in relation to quinine stimulation, we observed that the data closely resembled that of the behavioral data; nearly all wildtype animals responded in both behavioral and physiological experiments whereas less than half of *cat-2* animals responded. This trend continued as we treated the animals (both *cat-2* and wildtype animals) with exogenous dopamine. These results may indicate that although we observed an increase in the Ca^{2+} activity of the *cat-2* ASH neurons, this does not necessarily mean that this would in fact elicit a change in behavioral response. A trivial explanation for this observation is that the fraction of animals responding in the Ca^{2+} recordings is just an artifact caused by experimenter handling; this is possible, but we do not think this is the case. One possible explanation is the exogeneous dopamine we added may have restored Ca^{2+} flux amplitude but not probability of firing in ASH neurons. In such a scenario, exogenous dopamine would also have to activate other sensory neurons to detect quinine when ASH does not fire. *C. elegans* alters the sensory neurons used to detect volatile chemical repellants depending on serotonin levels (Chao et al., 2004), so this is not without precedent. Also, the ASK neurons are also known to be quinine sensing neurons (Hilliard et al., 2004). This implicitly suggests that dopamine must have multiple cellular sites of action; it is therefore useful to consider dopamine receptors.

Both *dop-1* and *dop-4* mutant strains were shown to respond to the quinine stimulus less frequently than wildtype animals in behavioral assays (See section 3.A.). Unlike *cat-2*, both mutant strains responded reliably in Ca^{2+} recordings (about 7-9 responses vs. 17-19 responses per 20 trials, respectively). Much to our surprise, the dopamine receptor mutants had far more robust Ca^{2+} responses, showing a ~250-400% increase in ASH activity in comparison to wildtype animals. The Ca^{2+} recording data suggests a down-regulatory role for dopamine receptors in the activity of the ASH neurons in response to quinine stimulation; but the behavioral results suggest that these dopamine receptors up-regulate ASH-mediated aversive behaviors (See section 1.H.2. & 3.A.). In other words, an increase in the activity of the ASH neurons does not necessarily equate to an increase in aversive behaviors.

The idea that dopamine might have multiple cellular sites of action is consistent with this discrepancy we observed in the *dop-1* and *dop-4* mutants. For instance, dopamine might dampen ASH activity, but dopamine might also activate other downstream neurons that promote behavioral output. In this scenario, deleting the dopamine receptors would result in a net decrease of behavioral output because of a downstream bottleneck, even though ASH Ca^{2+} activity is increased. Alternatively, Ca^{2+} activity in ASH neurons does not directly correlate with behavioral output. In this scenario, dopamine receptors also dampen ASH activity, but large increases in intracellular Ca^{2+} actually result in less neural output. For instance, ASH neurons also use neuropeptides as

neurotransmitters, and neuropeptides usually are released with increased presynaptic activity (Ezcurra et al., 2016). Admittedly, all this is difficult to reconcile with these results and those of the *cat-2* mutants with exogenous dopamine treatment.

As shown, our experiments support the hypothesis that dopamine plays a regulatory role on the ASH neurons, but a there does not appear to be a simple model that is consistent with all our data. Further understanding of this sensory-behavioral network could possible shed light on the complex activities of dopamine signaling. Investigation of the other neurons in communication with the ASH neurons could provide insight into dopamine regulation outside of the ASH neurons themselves. We could first use a series of transgenes expressing dsRNA targeted against *unc-13* using cell specific promoters. *unc-13* codes for a protein associated with stabilizing other pre-synaptic vesicle docking proteins (Richmond and Broadie, 2002) and knocking down *unc-13* activity by RNAi would block synaptic transmission(Grishok et al., 2005). By blocking synaptic transmission, the *unc-13* mutation would allow for the systematic silencing of individual neurons that might regulate ASH. Using our new understanding of the activity of ASH in regard to dopamine sensitivity and detection of quinine, both in terms of behavior and Ca^{2+} response, we could identify neurons in which lack of synaptic transmission would alter ASH Ca^{2+} response patterns. For instance, ADL sensory neurons receive synaptic input from the CEPV dopaminergic neurons and send synaptic output to ASH neurons (Fig. 1). Knocking down *unc-*

13 activity in CEPV or ADL might show if those connections are relevant in dopamine modulation of quinine response. Experiments such as this could help us understand the cellular location(s) of dopamine activity and regulation.

We still do not have a clear understanding of the dopamine receptor expression patterns. Based on functional studies we assume at least some of them to be expressed on the ASH neurons (Ezcurra et al., 2016), but direct expression evidence is still lacking. Dopamine receptor expression has been shown in many other head neurons (Chase et al., 2004; Sugiura et al., 2005; Suo et al., 2003), and that is consistent with the idea these dopamine receptors may likely be active in other neurons to regulate this behavior. To further characterize the sites of action, one could attempt cell specific transgenic rescue of dopamine receptor mutants. For instance, the *sra-6* promoter could be used to specifically rescue the dopamine receptors within the ASH neuron. This approach may allow for the understanding of which receptor is functioning in which neuron. Though the above-mentioned experiment could be useful in understanding the possible activity of these dopamine receptors in specific cells, it still does not describe the natural expression patterns of the dopamine receptors.

To better understand the natural expression patterns of dopamine receptors, our lab is currently utilizing CRISPR technology by replacing the *dop-4* and *dop-1* coding sequences with GFP reporter sequence in their natural chromosomal context (L. Velasquez Macedo, in preparation). It could be interesting to examine strains with multiple dopamine receptor mutants to

observe possible antagonistic relationships between the various dopamine receptor mutants. We observed that the D1-like DOP-1 and DOP-4 receptors act to down-regulate the activity of the ASH neurons, but we have yet to experiment with the *dop-2* and *dop-3* mutant strains. It is possible that the D2-like DOP-2 and DOP-3 receptors could act to up-regulate ASH activity and so may play an antagonistic role with the DOP-1 or DOP-4 receptors. Further examination of these dopamine receptor mutants may provide a better understanding of the complex regulatory roles of dopamine within sensory neural circuits.

APPENDIX A:

FIGURES AND TABLES

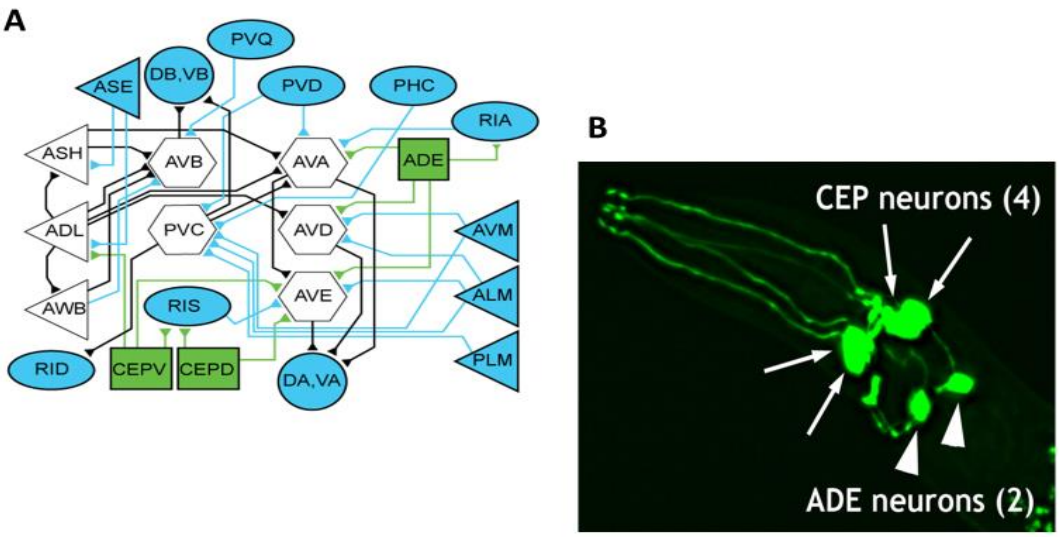

Figure 1: A circuit diagram of a group of *C. elegans* sensory neural circuits (A); triangular shaped neurons represent sensory neurons, hexagonally shaped neurons are interneurons, the neurons colored in blue are those that express one or all dopamine (DA) receptors, and the neurons colored greens are those that are dopaminergic in natural, that is they are cellular sites of DA production and release. GFP reporter assay show the expression pattern of tyrosine hydroxylase (B), a key enzyme in the pathway leading to the production of dopamine (Caldwell and Caldwell, 2008).

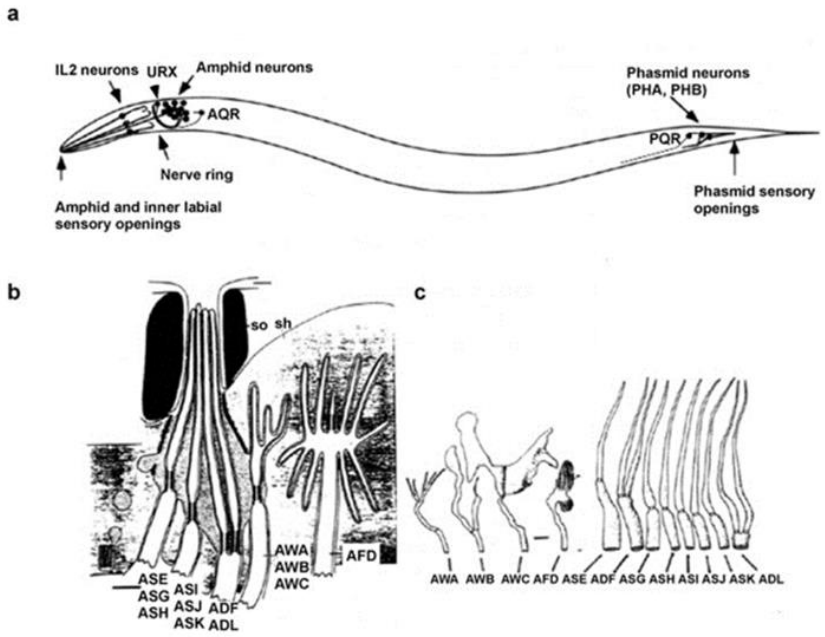

Figure 2: The amphid neurons of the C. elegans hermaphroditic animals are located within the head just posterior to the pharyngeal pump, these neurons (ASE, ASG, ASH, ASI, ASJ, ASK, ADF, ADL, AWA, AWB, AWC, AND AFD) represent the primarily sensory neurons of the animal; C. elegans express bilaterally symmetric amphid structures. There also exists a similar structure in the posterior end of the animals known as the phasmid, which contains sensory neurons (PHA, PHB, and PQR) as well (a). A more detailed illustration of the amphid pore reveals the structure and ordering of the sensory neurons (b); detailed illustrations show the structure of the amphid neurons dendritic sensilla (c) (Adapted from and reviewed in Bargmann, 2006).

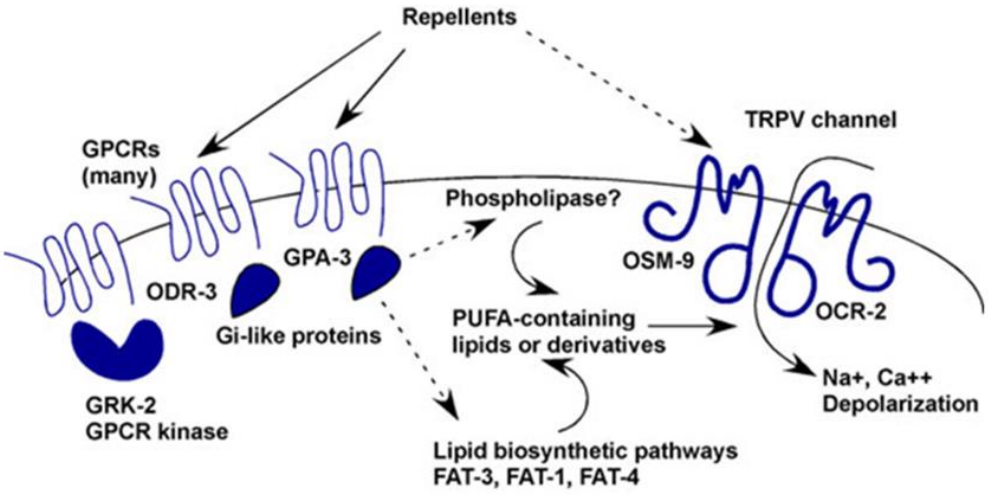

Figure 3: ASH sensory activity is likely driven by a number of cellular receptors. GPCRs are known to detect stimuli from the environment, in turn activating Gi like subunits within the cell. It is also possible that the TRPV channels themselves can be activated by external stimuli inducing a depolarization of the cell via the cation flux through open channels (Adapted from and reviewed in Bargmann, 2006).

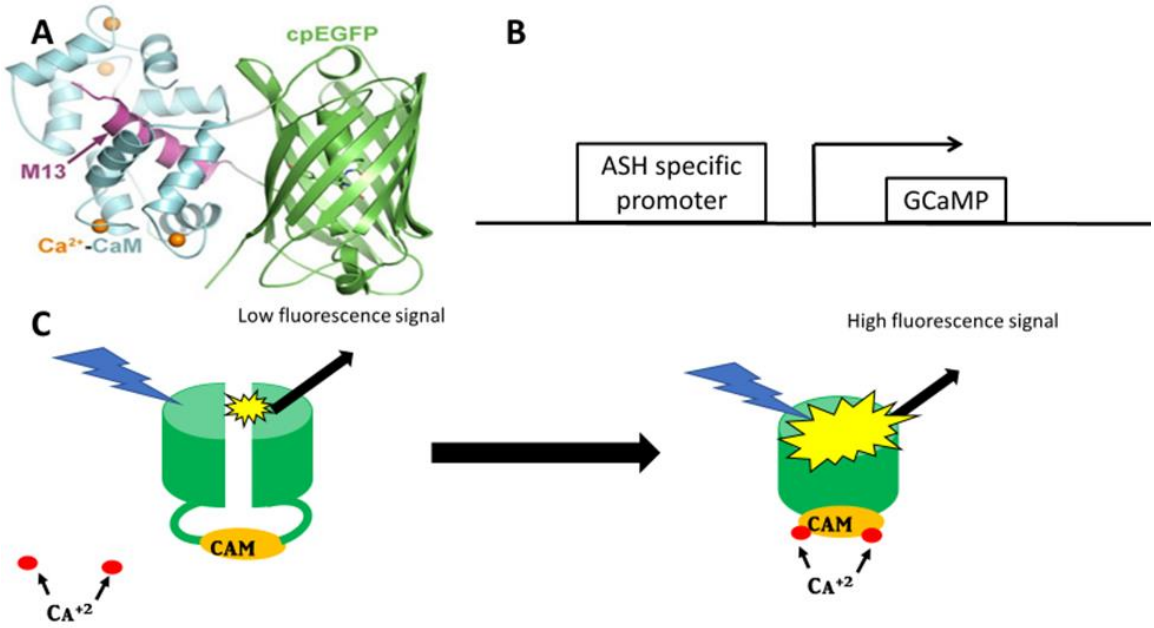

Figure 4: Genetically engineered Ca^{2+} sensitive calmodulin domain containing green florescent protein (G-CaMP) reporter proteins (Nakai et al., 2001) (A) allow for the quantification of neural activity within the *C. elegans* animals. The transgenically coded G-CaMP genes are expressed specifically in the ASH neuron (B). In regions of low Ca^{2+} concentrations the G-CaMP proteins fluoresce at a low intensity; the intensity increases proportionally to match increases in Ca^{2+} concentrations (C).

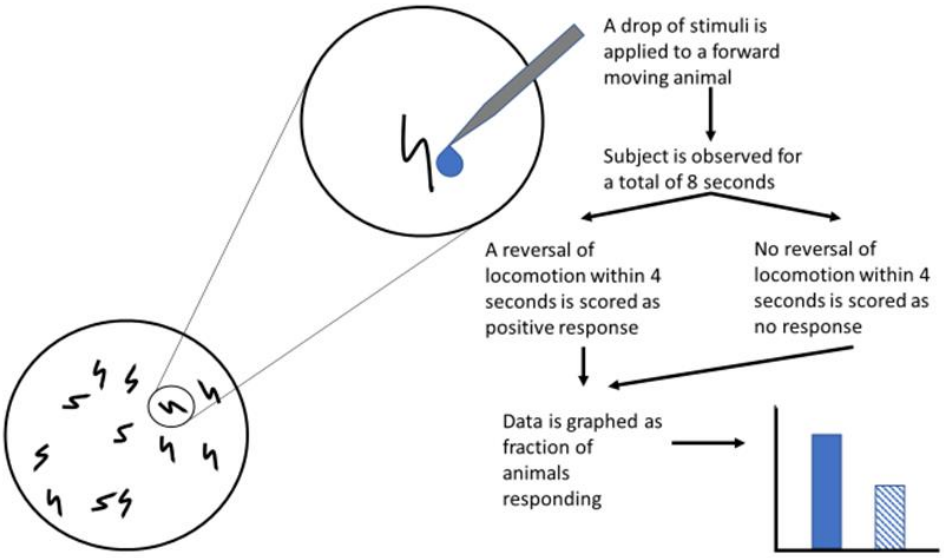

Figure 5: The drop test behavioral assay consists of a micro-droplet of repellent applied to a forward locomoting animal; responses are measured as a reversal of locomotion within a given time frame.

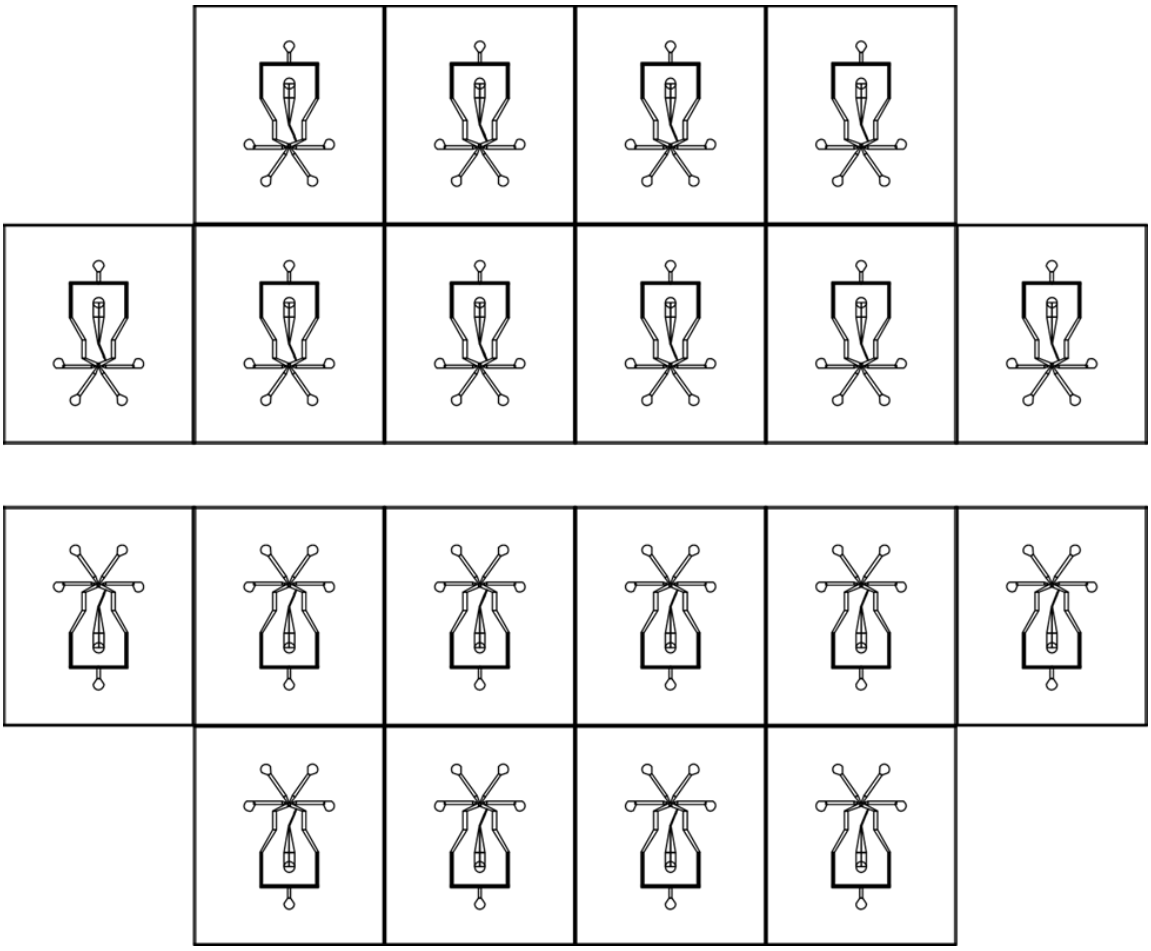

Figure 6: An image of the template used to create the spincast microfluidics chip mask (Chronis et al., 2007). As seen, one mask contains information for the casting of 20 microfluidics chip in a single molding. The microfluidics chips are cut apart prior to any further processing.

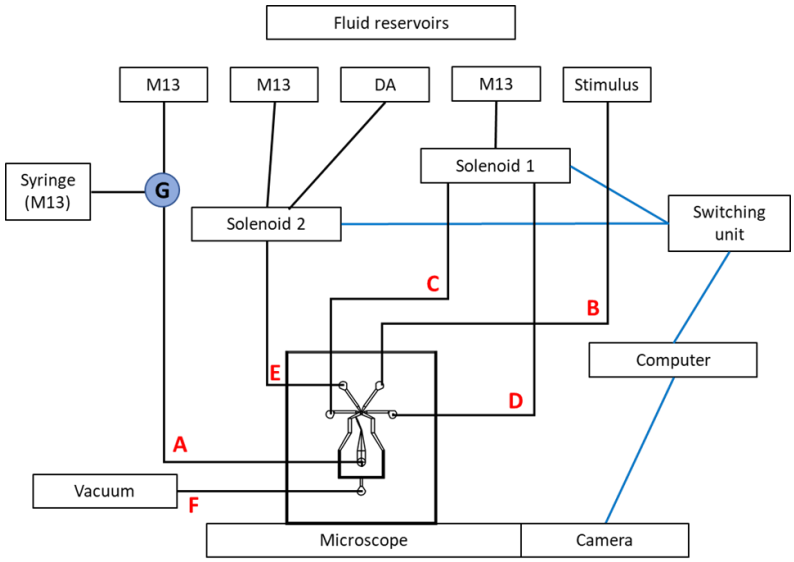

Figure 7: A diagrammatic figure of microfluidics device set-up, also pictured is an enlarged image of the microfluidics recording chip. The tubing marked “A” attaches to the chip at port 1; it is removed from the chip to pick up worms; this activity is achieved by the addition of a three-way Luer lock valve (G) controlling flow from the fluid reservoir (used to keep worm in position during recording) and the syringe used for manipulating the worms. The tubing marked “B” attaches to the chip at port 5; it delivers stimuli to the recording chip and is always placed to the opposite side of the worm trap. The tubing marked “C” attaches at port 3 and “D” attaches at port 6; tubing “C” and “D” are derived from the same fluid reservoir but utilize an electronic switching solenoid (1)1 to alter the flow of fluids with the recording chip provide rapid exposure and elimination of stimuli. The tubing marked “E” attaches to the chip at port 4; it is derived from two separate reservoirs, one containing M13 and the other the dopamine suspended in M13; the switching solenoid (2) has been run in reverse in comparison to solenoid 1.

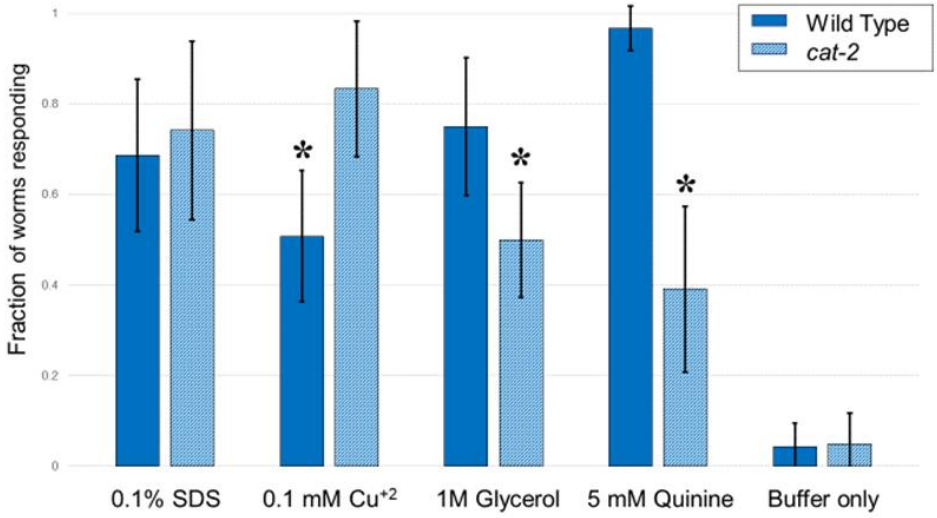

Figure 8: *cat-2* mutant animals respond differently than wildtype animals to water soluble stimuli. We found significant differences to the response of WT and *cat-2* dopamine deficient mutants to 0.1 mM Cu^{2+}, 1M glycerol and 5 mM quinine. These two strains exhibited no difference in response to either 0.1% SDS or the M13 buffer (negative control).

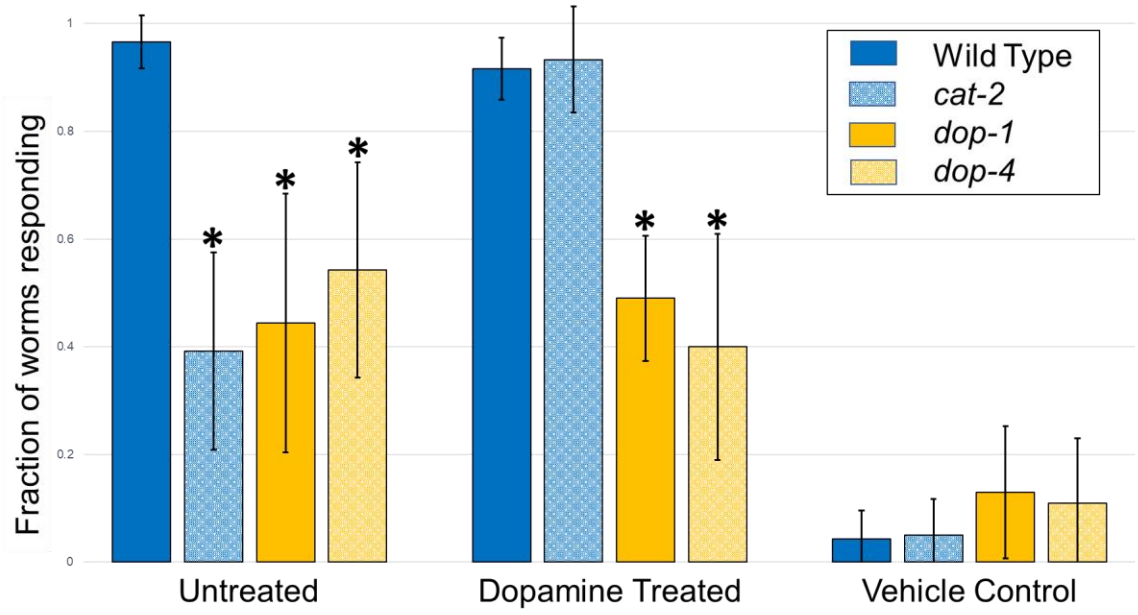

Figure 9: Exogenous dopamine restores normal behavior to *cat-2* animals stimulated with 5 mM quinine, shows no effect on the *dop-1* or *dop-4* animals (data obtained by J. Jensen). Worms were tested on NGM plates that had been treated to achieve 4 mM dopamine concentration, untreated plates and treated plates with an M13 negative control stimuli. We found significant differences in the untreated assays, yet no significant difference in the treated assays indicating a significant effect on the worms' behavior.

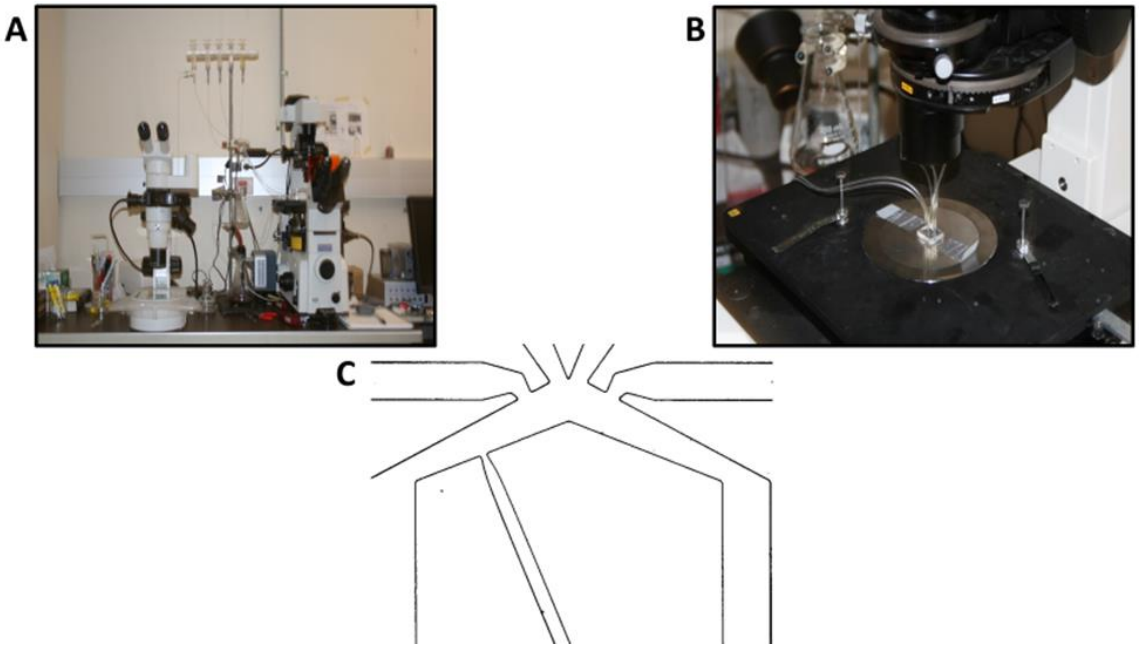

Figure 10: The microfluidics rig is set up to run over the inverted fluorescent microscope with an accompanying fluorescent dissecting microscope to enable selection and transfer of subject animals (A). A close up of the recording rig shows the tubing supplying the microfluidics chip with buffers and stimuli (B). The microfluidics chip has a total of 6 ports: 3 ports for buffer flow, one for stimuli, one for worm loading, and one for the vacuum line (C).

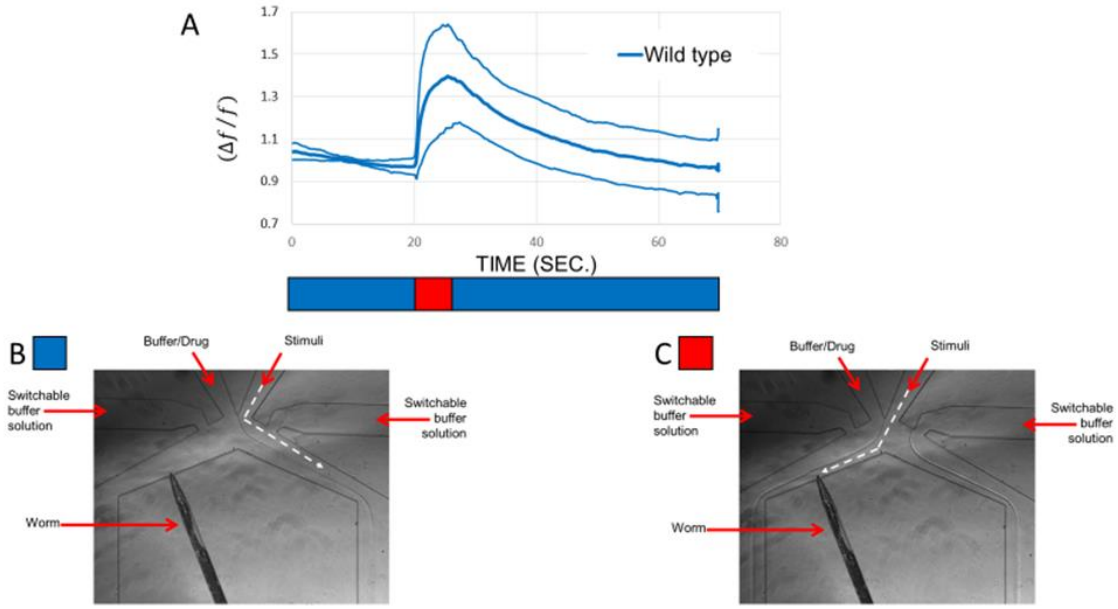

Figure 11: Transient Ca^{2+} recordings from the ASH neuron allow for the quantification of neural activity levels during stimulation. Data obtained during recordings can be averaged and normalized to create activity traces (A). An individual *C. elegans* animal is held in place within the microfluidics chip in such a way as to have the rostral end of the animal exposed to the fluid streams; in this position, the animal can be exposed to the M13 buffer (B) or to an aqueous solution, drug or stimuli (C).

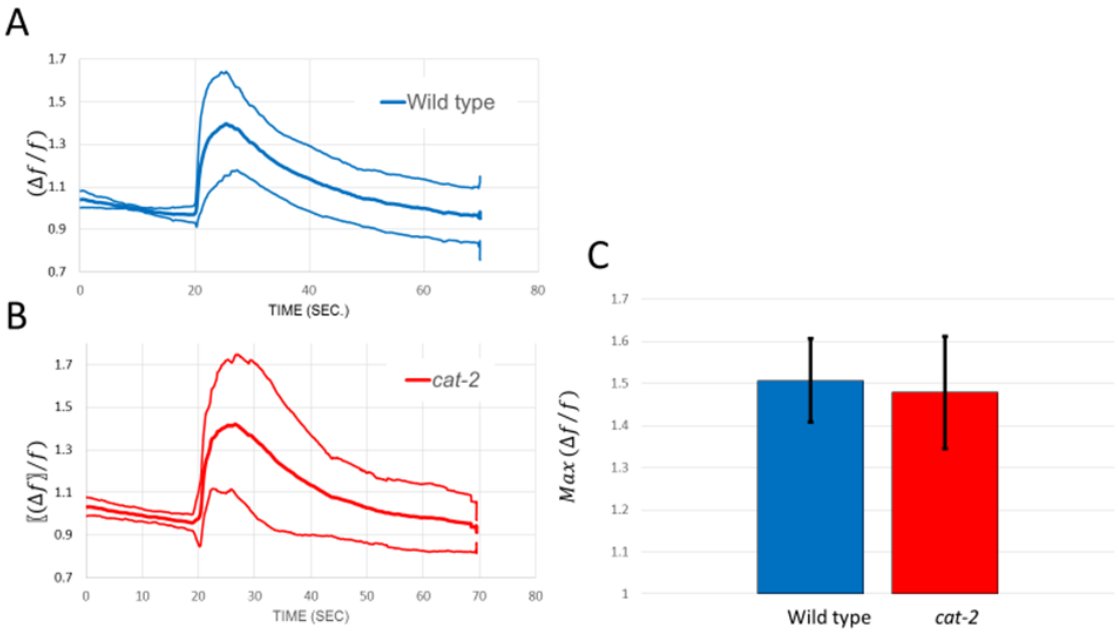

Figure 12: Using glycerol as a stimulus we characterized the response of the ASH neurons in both wildtype (A) and *cat-2* (B) animals; bold lines are that of the average response (n=10) and the thinner lines that of +/- the standard deviation. We reported no significant difference in the maximum intensity of response between the two strains (C).

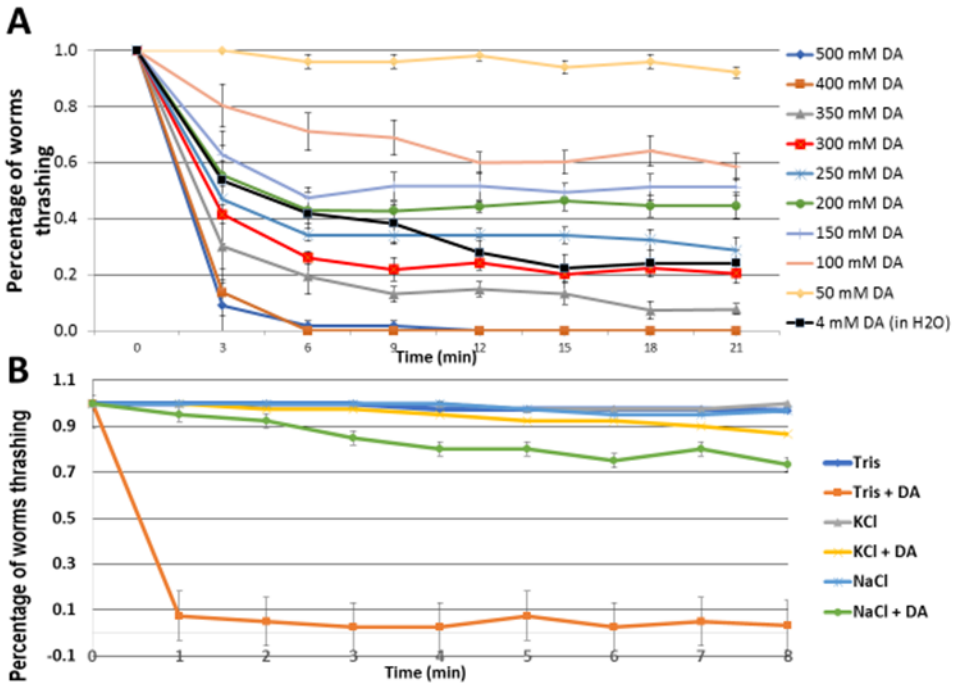

Figure 13: Dopamine (DA) renders *C. elegans* animals paralyzed during a SWIP assay. However, when dopamine is suspended in M13 recording buffer this paralysis dissipates (A). The salts found in the M13 recoding buffer disrupt the paralyzing activity of dopamine during the SWIP assay (B) (M. Torres and J. Kim).

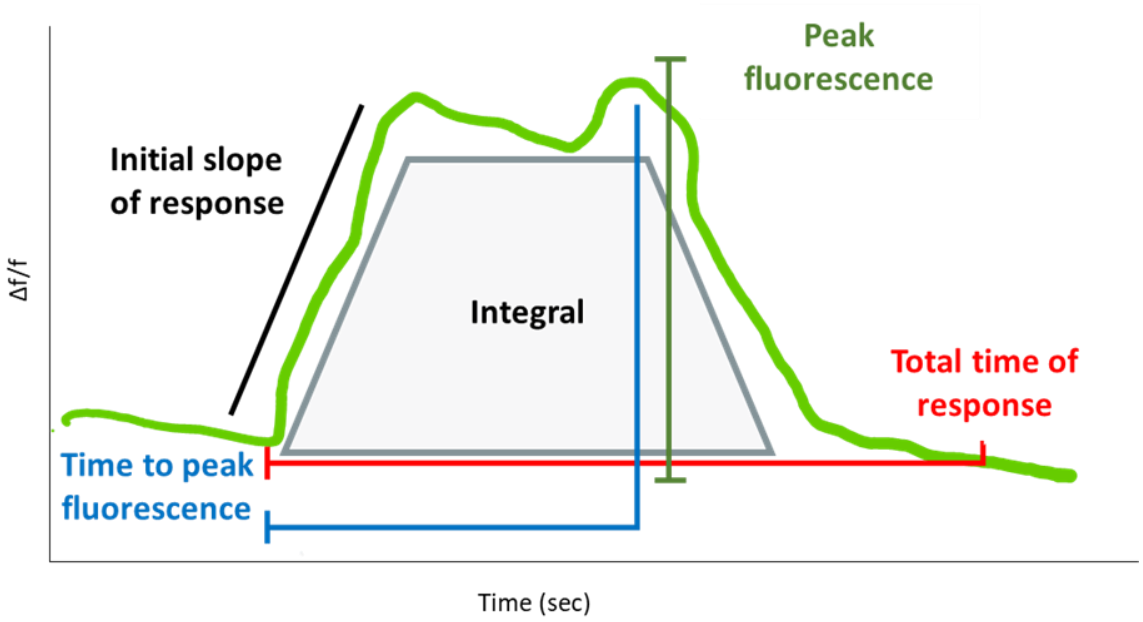

Figure 14: An illustration of the metrics measured with the obtained Ca^{2+} recordings. The dark green represents the measure of peak fluorescence, Δf/f is a relative measure of the increase in fluorescence over that of the baseline fluorescence. The red color represents the total time of response as far as the total time that fluorescence is above baseline. The blue color shows the measure of time from stimuli application to peak fluorescence. The black color shows the measure of the initial slope to the response trace. Lastly, the grey trapezoid represents the integral or the area found under the trace.

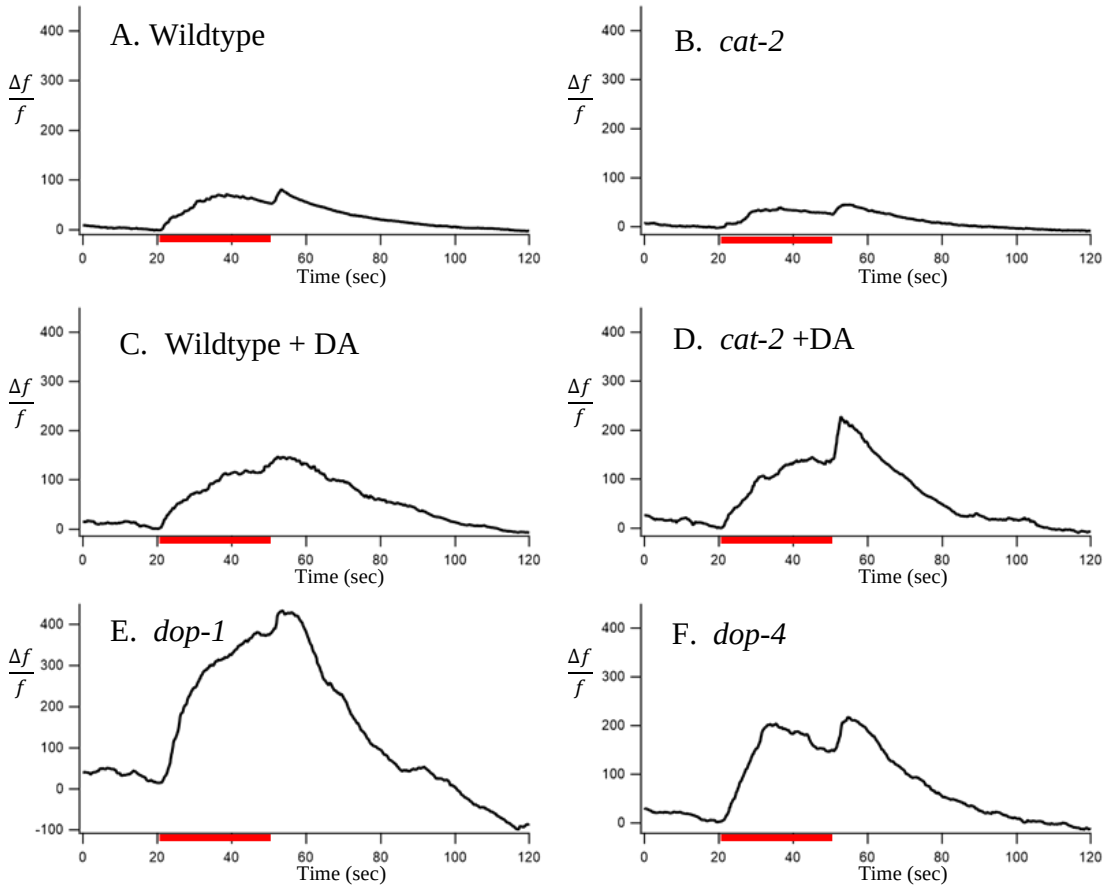

Figure 15: ASH neuron Ca^{2+} changes in response to 50 mM quinine stimulation. The red bar indicates period of stimulation (30 sec). Ca^{2+} response trace of (A) wildtype animals (n=17/20; fraction of animals responding versus tested), (B) *cat-2* animals (n=7/20), (C) wildtype animals treated with 4 mM exogenous dopamine (+DA) (n=13/20), (D) *cat-2* animals treated with 4 mM exogenous dopamine (n=9/20), (E) *dop-1* animals (n=19/20), and (F) *dop-4* animals (n=17/20).

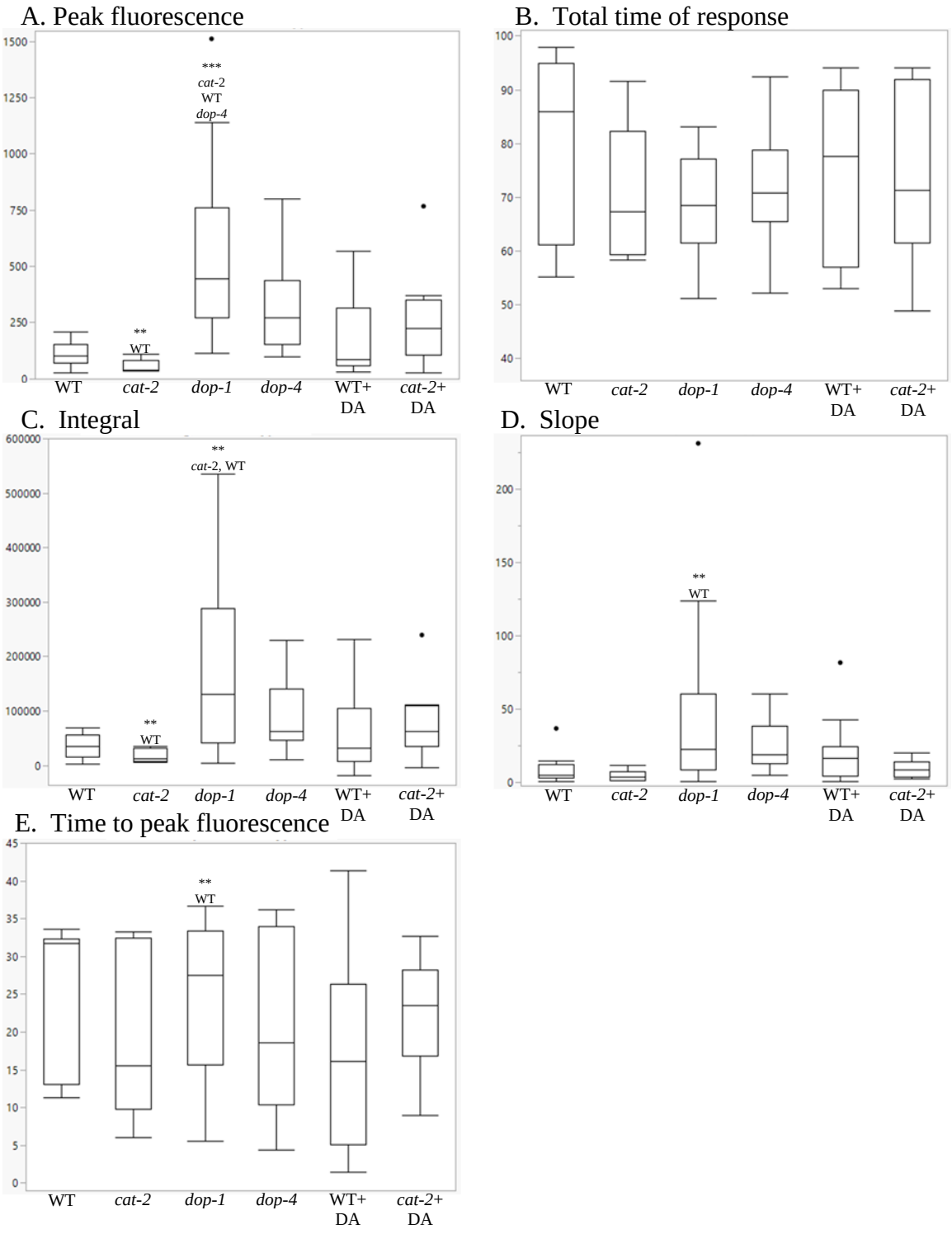

Figure 16: legend on following page.

Figure 16: (previous page) T-test and ANOVA statistical analysis of the ASH response to 50 mM quinine in a variety of genetic backgrounds. Dots indicate outliers but are included in the analysis. (A) We observed a significant difference in the peak fluorescence of the Ca^{2+} traces between the (n=17/20; fraction of animals responding versus tested) wildtype and (7/20) *cat-2* animals (p=0.0118). ANOVA (α≤0.05) revealed a difference among wildtype *cat-2*, *dop-1* and *dop-4* (F<0.0001). The Tukey-Kramer method of comparison identified significant differences between *cat-2* and *dop-1* animals (p=0.0003), between wildtype and (n=17/20) *dop-1* animals (p<0.0001), and between *dop-1* and (n=19/20) *dop-4* animals (p=0.0343). (B) We observed no statistical difference in the total time of response of the Ca^{2+} traces. (C) We observed a statistical difference in the integral of the Ca^{2+} traces between *cat-2* animals and wildtype animals (p=0.0162). ANOVA (α≤0.05) indicated significant differences among wildtype, *cat-2, dop-1,* and *dop-4* (F=0.0016). The Tukey-Kramer method of comparison identified significant differences between *cat-2* and *dop-1* animals (p=0.0081), and between wildtype animals and *dop-1* animals (p=0.0064). (D) ANOVA (α≤0.05) indicated a significant difference among wildtype, *cat-2, dop-1,* and *dop-4* (F=0.0181). The Tukey-Kramer method of comparison identified a statistical difference in the initial slopes of the Ca^{2+} traces between wildtype animals and *dop-1* animals (p=0.0370). (E) ANOVA (α≤0.05) indicated significant differences among wildtype, *cat-2, dop-1,* and *dop-4* (F=0.0694). The Tukey-Kramer method

of comparison identified a significant difference in time to peak fluorescence between *dop-1* and wildtype animals (p=0.0487).

Primer name	Sequence (5'-3')	Orientation	Strain	Notes
cat-2 #4	gcgaatgacgtcactcctatcg	sense	*cat-2*	left primer for *e1112*
cat-2 #3	gagacatatgagctagcagtgg	antisense	*cat-2*	right primer for *e1112*
oZY46	cagctctaaaacaattatggttcgt	sense	*dop-1*	left primer for *vs101*
oZY47	catatcttgacgagctaaatttcca	antisense	*dop-1*	internal primer for *vs101*
oZY48	acagaaacagatgaaaaatgagacc	antisense	*dop-1*	right primer for *vs101*
oZY55	aatgttggcttacgggtctg	sense	*dop-4*	left primer for *tm1392*
oZY56	aaaattaggtctcggcacagc	antisense	*dop-4*	internal primer *tm1392*
oZY57	caaggcacgttctagtgcag	antisense	*dop-4*	right primer for *tm1392*

Table 1: identification of all primers used for genotyping the various mutant strains used in this study. Primer names are specific to our lab. Sense/antisense is relative to the target gene coding sequence.

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