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Elucidating the role of dopamine in the avoidance response of Caenorhabditis Elegans utilizing microfluidics

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ELUCIDATING THE ROLE OF DOPAMINE IN THE AVOIDANCE RESPONSE OF *CAENORHABDITIS ELEGANS* UTILIZING MICROFLUIDICS

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

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in

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ABSTRACT

Dopamine is an important neurotransmitter that is utilized in a wide range of phyla, from nematodes to mammals. Within humans, dopamine is known to be involved in many important processes, including learning and cognition, drug addiction, and Parkinson's disease. Because nematodes such as *Caenorhabditis elegans* utilize dopamine at the molecular and cellular level in much the same way as mammals, they offer researchers a unique opportunity to study the mechanisms of dopamine signaling. This study looks at dopamine modulation of the ASH neurons, which are part of the avoidance circuit of *C. elegans,* and their response to water soluble stimuli. The avoidance response to the detergent SDS and the high osmolarity stimulus glycerol were found to be modulated by dopamine, while response to the bitter tastant quinine was not. In order to visualize activity of the ASH neuron a microfluidics-based fluorescent imaging system was established. This system allows for direct visualization of neuronal activity as well as precise control over exposure of the worm to stimulus. Microfluidics experiments were then utilized to visualize activity of the ASH neuron in response to exposure to glycerol. Mutant animals deficient in dopamine biosynthesis were found to have an increased response to the removal of glycerol as compared to dopamine deficient mutants. My results suggest that dopamine signaling may potentiate ASH neural activity, although the direct site of action remains to be determined. The recording methodology I established will greatly facilitate future analysis of *C. elegans* neural activity in the lab.

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CHAPTER ONE

INTRODUCTION

1.1 Dopamine is an Important Neurotransmitter Utilized by a Wide Range of Phyla

Dopamine is an important neurotransmitter known to be involved in locomotion, cognition, learning, and the disease phenotypes of Parkinson's disease and drug addiction (Chiara, 1999; Lotharius & Brundin, 2002; Schultz, 2006; Wise, 2004). There has therefore been a great deal of interest in determining the cellular and molecular mechanisms of dopamine signaling in the human brain. Model organisms such as the nematode *C. elegans* have been utilized to study dopamine function. Dopamine is evolutionarily conserved across a wide variety of phyla from invertebrates, such as *C. elegans* (Sulston et al., 1975) , to vertebrates, such as human beings.

1.11 Most Dopamine Receptors are G Protein Coupled Receptors

The neurotransmitter dopamine is sensed by dopamine receptors on the plasma membrane of cells. These receptors are G-protein coupled receptors (GPCRs). GPCRs are characterized by seven hydrophobic transmembrane regions that allow an extracellular signal to be transmitted inside the cell. Within the cell, GPCRs are bound to a heterotrimeric G protein, an inner membrane peripheral membrane protein that is composed of an alpha, beta, and gamma subunit. The alpha subunit is also bound to guanosine diphosphate (GDP). Upon binding of the GPCR with its ligand the GPCR will change conformation, which

activates the heterotrimeric G protein. Upon activation GDP is replaced by GTP. This triggers the release of the G protein from the GPCR. The heterotrimeric G protein will then disassociate into two smaller subunits which can both go on to trigger the production of secondary messengers. One common secondary messenger is cyclic adenosine monophosphate (cAMP). cAMP can activate a signal cascade that can result in a number of different physiological responses depending on the cell type in which it occurs (Figure 1).

There are five types of dopamine receptors that are divided into two major groups: D1 and D2 receptors. D1 receptors activate the $G\alpha_{s/olf}$ pathway, which activates the adenylate cyclase enzyme that produces cAMP (Hemmings et al., 1984; Kebabian & Calne, 1979). The D2 receptors activates the $G\alpha_{i/o}$ pathway, which inhibits adenylate cyclase and therefore reduces the levels of cAMP (Caron et al., 1978; Kebabian & Calne, 1979).

Although most dopamine receptors are GPCRs, recently there were found to be dopamine gated chloride channels in *C. elegans* (Ringstad et al. 2009). However, their biological function remains unclear.

1.12 Dopamine Plays a Role in Impulse Control Disorders

Dopamine signaling is thought to play important roles in disease phenotypes in the human brain, such as drug addiction and Parkinson's disease. For instance, it has been shown that drugs such as amphetamines cause a marked increase in dopamine levels within the synaptic cleft of the neurons within the striatum (Zetterström, et al., 1983). Parkinson's disease patients

offered researchers a unique situation in which the response to increased dopamine signaling could be investigated in humans.

Parkinson's disease is a disorder that is, in part, characterized by uncontrollable movement of the body. This uncontrolled movement is due to insufficient dopamine signaling within the substantia nigra (Damier et al., 1999). One common treatment for this disorder is administration of either levodopa, which is the biosynthetic precursor to dopamine, or a dopamine agonist such as pramipexole. These drugs work to increase dopamine levels, which then reduce the symptoms of the movement disorder. Treatment with dopamine agonists sometimes resulted in the patient engaging in pathological gambling, compulsive shopping, compulsive eating, addiction to the medication, or hyper sexuality, all known collectively as impulse control disorders. This finding was especially interesting as these behaviors are contrary to the personality of normal Parkinson's patients, who usually exhibit the so called Parkinsonian personality, which is characterized as rigid, introverted, and slow to anger (Todes and Lees, 1985). It has also been noted that those with Parkinson's disease show little novel seeking behaviors (Leyton et al., 2002), which is thought to result in lower risk of addiction (Evans et al., 2006a). Among the patients who developed these dopamine agonist-induced disorders it was found that onset was usually within two months of beginning dopamine agonist treatment and ceased within a month of stopping treatment (Evans et al., 2006b). If, however, the patient's medications were changed such that their dose of dopamine agonist was decreased with a

concomitant increase in their levodopa dose the impulse control disorders would dissipate (Mamikonyan et al., 2008). This suggested that an increase in dopamine signaling is at least partially responsible for so-called impulse control disorders, which include drug addiction.

What part of the brain does dopamine work on to cause these impulse control disorders? The ventral striatum had been implicated in drug addiction (Robbins and Everitt, 1999). This hypothesis is strengthened by the findings that Parkinson's patients given levodopa are found to have improved performance on cognitive tasks mediated by the dorsal striatum, whose signaling is greatly affected by Parkinson's, and lowered performance in reversal learning, which is mediated by the ventral striatum. This decreased reversal learning is thought to be the result of excessive transmission within the ventral striatum due to increased dopamine levels, while the increased cognitive functions is due to the drug levodopa supplementing the dopamine lost during the course of the disease (Cools et al., 2001). However, it has not yet been shown that this increased transmission in the ventral striatum is involved in impulse control disorders.

One of the hallmarks of continued drug abuse is sensitization, which is characterized by increased response to constant doses of a stimulant drug upon repeated administration. It was found that among the Parkinson's patients exhibiting impulse control disorders, all had significantly higher dopamine release in response to levodopa administration in the ventral striatum, but not the dorsal striatum (Evans et al., 2006b). This increased dopamine response is similar to

the response seen when healthy adults are given amphetamines (Leyton et al., 2002). This suggests that dopamine treatment mimics the sensitization to prolonged drug usage. It had also been previously established that the amount of dopamine released correlated with the reported want for the drug; individuals with higher levels of dopamine transmission wanted to take the drug again more as compared to those with lower levels of dopamine transmission (Boileau et al., 2006). This suggests that increased levels of dopamine caused by treatment with levodopa results in increased dopamine transmission, which mimics the response of sensitization to a drug, and that dopamine treatment can elicit responses in patients similar to those in individuals who had become sensitized due to drug use. Thus, increased dopaminergic transmission within the ventral striatum might be involved in the emergence of the impulse control disorders.

1.2 *Caenorhabditis elegans* is a Model for Neurobiology Research

Dopamine signaling can be studied using a wide array of model organisms including rats, mice, and primates to invertebrates such as *Drosophila* and *C.elegans*. *C. elegans* is a free living self fertilizing hermaphrodite and is almost exclusively found in anthropogenic habitats, such as garden soil and compost. They are known to associate with a number of different arthropods such as millipedes, slugs, and snails. In the lab a single animal can produce hundreds of progeny in a period of three to five days depending on the cultivation temperature

(Figure 2). In the lab they feed on *E. coli* bacteria, which itself is grown on agar dishes alongside *C. elegans.* It is useful in behavioral chemosensory studies as it responds to different chemical attractants and repellants. Mutant strains, which lack or over express certain genes and proteins, can be made relatively easily and their behavior can be compared to wild type in order to determine the function of these genes and proteins.

C. elegans is also very well suited to direct study of neuronal circuits. All 302 *C. elegans* neurons have been identified. Neurons are named with three to four letters indicating class. If the neuron is bilaterally or dorsoventrally symmetrical an R (right), L (left), D (dorsal), or V (ventral) is added to the end of the designation. All of the synaptic connections have been mapped down to the synaptic level (White et al., 1986). Manipulation of this nervous system will often result in behavioral changes that can be characterized and quantified. Of its 302 neurons, sixteen pairs of bilaterally symmetrical neurons are thought to be involved in chemosensation (Bargmann et al., 1993). Of these sixteen pairs, eleven are expressed at the anterior portion of the worm and eight express ciliated sensory endings that extend through the cuticle via a structure called the amphid pore to sense the environment (White et al., 1986) (Figure 3). One of these is the ASH neuron pair, which is one of the primary neurons of interest in this proposal.

ASH is a polymodal neuron that detects noxious stimuli. It is known to sense not only water soluble stimuli, but volatile, mechanical, and osmotic stimuli

as well. For instance, when the osmolyte glycerol is sensed by ASH in a forward moving animal there is a tonic increase in intracellular Ca^{2+}concentration. Upon removal of the stimulus, the ASH neuron responds with a transient increase in intracellular Ca^{2+} that is equal to, or greater than, the response to the initial exposure (Chronis et al., 2007). This signal is then passed from the ASH neuron downstream to command interneurons such as AIY, AIA, AIB, AVA, and AVB (White et al., 1986). These interneurons then go on to innervate the muscle, which results in a backward locomotory response (i.e., a reversal). For instance, *C. elegans* cannot escape a ring of glycerol drawn on an agar surface, due to ASH-mediated reversals of locomotory direction. ASH neurons also mediate reversals when it detects quinine, SDS, copper (Hilliard et al., 2004a), octanol (Troemel et al., 1997), or nose touch (Kaplan and Horvitz, 1993).

The ASH-mediated avoidance response can be modulated by certain amines such as serotonin, dopamine, tyramine, and octopamine. The aversive response is known to increase in the presence of serotonin (Chao et al., 2004). This is believed to be due to 5-HT receptors expressed on the AIA, AIB, and RIA interneurons as well as on the ASH sensory neuron (Harris et al., 2009). Therefore, serotonin acts upon multiple neurons at different levels within the neural circuit. Tyramine and octopamine are known to counteract serotonin's effect on the ASH response. This response is mediated by two different GPCRs, TYRA-3 and F14D12.6, that recognize tyramine and octopamine, respectively. F14D12.6 was found to be expressed in the ASH neuron, while TYRA-3 was

found to be expressed in the head and tail neurons, vulval muscles, and APE and CDE dopaminergic neurons. Although tyramine affects aversive response similarly to dopamine (see below), and its receptor is found on dopaminergic neurons, it was found that the role of dopamine is separate from the effect of tyramine on aversive response (Wragg et al., 2007). *C. elegans* uses dopamine to regulate a wide range of behaviors. In addition to the avoidance responses described above, these also include: modulating locomotory rate in response to food, mechanosensory plasticity, and food searching. Below, I review some of the studies on dopamine-modulated behaviors in *C. elegans* that highlight the importance of dopamine in the *C. elegans* nervous system.

1.21 Dopamine Modulates Locomotory Rate in Response to Food

Organisms are often faced with environments where food is in short supply, and they must therefore maximize their ability to locate and remain near a food source. When C. elegans comes into contact with a food source, such as a bacterial lawn, the worms tend to slow their rate of locomotion. This behavior is referred to as the basal slowing response (Sawin et al., 2000). The cat-2 mutants, which are defective for the gene coding for the dopamine biosynthetic enzyme tyrosine hydroxylase (R. Lints, 1998), do not exhibit a basal slowing response. Pharmacological rescue experiments found that the basal slowing response could be restored in the cat-2 mutants when tested in the presence of 2 mM exogenous dopamine. These results indicated that dopamine is important in this process.

To investigate which dopaminergic neurons were responsible for this modulation, a series of laser ablations were performed on the eight dopaminergic neurons present in the *C. elegans* nervous system: the four CEPs, the two ADEs, and the two PDE neurons. Laser ablations are often used in *C. elegans* neurobiology to remove a single neuron within the nervous system. Upon brief exposure to laser pulses, the neuron will be killed without affecting adjacent cells (Bargmann and Avery, 1995). These studies indicated that not only did all three dopaminergic neuron types release dopamine in response to food, but that they all work redundantly to mediate the basal slowing response in the presence of food.

Dopamine is likely released in response to mechanical and not olfactory or gustatory stimulation from the bacteria. Worms moving through the Sephadex beads showed slowing similar to when the worms were in the presence of bacteria. Sephadex beads which are about 20-50 μM in size and are too large to be ingested by the worm; they are also presumably odorless. Furthermore, when the ADE and PDE dopaminergic neurons on one side of the body were ablated, and that side was in contact with food, the animals would not exhibit a basal slowing response. Conversely, if the side with the ablated neurons was not in contact with the bacteria the animals would exhibit a normal basal slowing response. Thus, it is the "feel" of the food rather than the smell or taste that elicits dopamine release (Sawin et al., 2000). This role of dopamine is extremely important to *C. elegans* as it ensures that they stay within an area in which food

is present. The fact that this system works through mechanosensation and not through chemosensation may indicate that while smell brings the worms to the food the feel of the bacteria indicates to the worm that it is physically located in the food.

Five dopamine receptors are encoded by the *C. elegans* genome. DOP-1 is a D1-like dopamine receptor (Suo et al., 2002), and DOP-2 is a D2-like dopamine receptor (Sugiura et al., 2005),. A novel receptor, designated as DOP-3, exhibits certain characteristics common to D2-like receptors (Chase et al., 2004). While animals lacking *dop-1* or *dop-2* showed no defects in basal slowing response, *dop-3* mutants did not slow down in response to food. When a wild type copy of the *dop-3* gene was transgenically introduced to *dop-3* mutant animals, normal basal slowing response was restored. The behavioral defect of *dop-3* mutants was similar to that of c*at-2* mutants, which are defective for dopamine production. However, the *dop-1;dop-3* double mutant exhibited a normal basal slowing response. This suggests that in the wild type worm the DOP-1 receptors activity antagonizes that of the DOP-3 receptor, and that the DOP-3 receptor likely inhibits locomotion while the DOP-1 receptor promotes it (Chase et al., 2004).

High concentrations of exogenous dopamine produce paralysis in wild type worms (Schafer & Kenyon, 1995), suggesting that dopamine can act extrasynaptically and/or humorally. The *dop-3* mutant animals show decreased sensitivity to high concentrations of exogenous dopamine, whereas *dop-1* or *dop-*

2 mutants do not. The *dop-1 dop-3* double mutant shows sensitivity similar to that of the wild type worms. This suggests that exogenous dopamine induces paralysis via a DOP-3 mediated pathway, and that the DOP-1 receptor works antagonistically to DOP-3 to decrease paralysis in wild type worms (Chase et al., 2004). Although the DOP-1 and DOP-3 receptors are both expressed on GABAergic and cholinergic motor neurons, DOP-1 and DOP-3 expression in the cholinergic neurons is most important to the basal slowing response. DOP-1 receptors mediate increased locomotion via the $G\alpha_q$ pathway, while the DOP-3 receptor mediated decreased locomotion via the $G\alpha_o$ pathway (Chase et al., 2004).

1.22 Dopamine Modulates Area Restricted Search

When an organism exhausts a food source it will often begin looking for another source close by. This behavior is called area restricted search (ARS). Most organisms do this by increasing their turning rate in order to stay relatively close to the initial food source (Karieva, 2013). C. elegans also exhibits this behavior and dopamine is important in this process. ARS is characterized by a high rate of turning after the worm has just been removed from a food source. This high turning frequency is presumably how the animals try to stay within an area in which food had already been found. If, however, the worm has not found food after searching for a prolonged period of time the worm will exhibit lower turning frequency in order to move to a new area. Wild type worms have a higher turning frequency when they have just been removed from a food source, and

this turning frequency decreases over time. Upon re-exposure to the food a reset of the ARS behavior occurs, such that after the worms are once again removed from food their turning rate is restored to initial levels. Worms have to physically move through food for the ARS behavior to reset, as dopamine is believed to be important in sensing food via mechanosensation in C. elegans as described above. Indeed, laser ablations of dopaminergic neurons resulted in animals that no longer exhibited the normal area restricted search behavior, but instead turned with a lower frequency after removal from food. Furthermore, wild type ARS behavior could be completely restored in ablated worms as well as cat-2 mutants with the administration of exogenous dopamine. Dopamine signaling, therefore, likely plays a prominent role in modulating this behavior (Hills et al., 2004).

1.23 Dopamine Modulates Mechanosensory Plasticity

Dopamine and the DOP-1 receptor are also involved in a certain type of mechanosensory plasticity known as tap habituation in *C. elegans* (Rankin et al. 1990). Tap habituation is a simple form of learning in which an animal will decrease its response (usually a switch from forward to backward locomotion) to repeated non localized taps or vibrations of the growth media. Both *cat-2* and *dop-1* mutants habituate faster than wild type animals, indicating that dopamine modulates the rate of habituation to non-localized tap stimuli via the DOP-1 receptor. Expression of a wild type copy of the *dop-1* gene in the ALM and PLM touch neurons was sufficient to restore the wild type habituation phenotype to

dop-1 mutants, suggesting that dopamine works through the dopamine receptor DOP-1 in the PLM or ALM neurons to modulate habituation (Sanyal et al., 2004).

The cytosolic Ca^{2+} content was measured in the ALM and PLM touch neurons of both wild type and various dopamine mutants using the Ca^{2+} reporter cameleon. This genetically-encoded Ca^{2+} indicator contains a calmodulin (CaM) domain that binds calcium, the calmodulin binding peptide of myosin light chain kinase (MLCKp), and a cyan fluorescent protein (CFP) as well as a yellow fluorescent protein (YFP). Because the two fluorescent protein have different excitation wavelengths they will not both fluoresce at the same time. When this molecule binds calcium it will undergo a conformational change such that CFP and YFP will come into close proximity; this results in fluorescent resonance energy transfer (FRET), which allows for the CFP to donate photons to the YFP, thereby allowing the YFP molecule to fluoresce at CFP's excitation wavelength. Changes to intracellular Ca^{2+} levels are therefore measured by the relative ratio of CFP to YFP (Miyawaki et al.,1997). In the ALM neurons (but not the PLM neurons), the magnitude of the Ca^{2+}transients in both the *dop-1* and *cat-2* mutants is initially very similar to wild type; however, Ca^{2+} levels decrease at a faster rate. This suggests that the faster habituation time exhibited by these mutants is in part due to decreased response to the stimuli in ALM (Kindt et al., 2007).

A rescue experiment, in which the *dop-1* gene was restored to ALM touch receptor neurons, showed that expression of the *dop-1* gene was sufficient to

restore the wild type phenotype. Mutants that did not express proteins involved in the $G_q\alpha$/PLC-β signal transduction pathway recapitulated the fast habituation of the *dop-1* mutants. An important component of the $G_q\alpha$/PLC-β pathway is the secondary messenger Ca^{2+}, which is released from the endoplasmic reticulum. Mutants that hindered normal Ca^{2+} release exhibit a fast habituation phenotype. Therefore, Ca^{2+} release from the endoplasmic reticulum in ALM neurons is essential for appropriate habituation response (Kindt et al., 2007).

The presence or absence of food is important to the tap habituation response. A wild type worm that is off food will show the fast tap habituation response of the *dop-1* mutant on food (Kindt et al., 2007). The dopaminergic neurons involved in this pathway are the ADE and CEP neurons, which are both known to detect the presence of food (Sawin et al., 2000). The *trp-4* mutants, which lack a TRPN channel important for sensing food in the CEP and ADE dopaminergic neurons (Walker, 2000; Li et al.,2006), show a fast habituation phenotype when tested on food. This could be rescued by expression of the *trp-4* gene in the ADE and CEP dopaminergic neurons. Ca^{2+} transients in the ALM neurons decay more rapidly in *trp-4* mutants than wild type animals, similar to *cat-2* and *dop-1* mutants (Kindt et al., 2007)

Taken together, the TRP-4 channel is likely involved in the activation of the dopaminergic neurons CEP and ADE in response to food. These neurons then release dopamine, which acts on the DOP-1 receptors on the surface of the ALM neurons. The DOP-1 receptor will then activate the $G_q\alpha$/PLC-β pathway

within the ALM, which causes release of Ca^{2+} into the cytosol from the ER. The ALM neuron can then act on interneurons and subsequently on motor neurons to modulate tap habituation. In this way, the worms are able to integrate information on stimuli that could indicate danger nearby such as tap or touch, with the presence of food (Kindt et al., 2007). (Figure 4).

1.24 Dopamine Modulates Sensitivity to Olfactory Stimuli

Dopamine has also been shown to affect the C. elegans response to olfactory stimuli. The aforementioned sensory neuron ASH is known to be involved in the avoidance response of C. elegans (Hilliard et al., 2004; Kaplan & Horvitz, 1993; Troemel et al.,1995). A recent study showed that the D2-like dopamine receptor DOP-3 may be both necessary and sufficient to the normal dopamine response of the sensory neuron ASH to the repellant octanol (Ezak and Ferkey, 2010).

The RGS protein RGS-3 modulates ASH GPCR signaling by accelerating the hydrolysis of GTP to GDP (Ferkey et al., 2007). The loss of this protein results in increased ASH signaling, which in turn causes increased sensitivity to octanol. However, normal sensitivity could be restored to *rgs-3* mutant animals if they were exposed to exogenously supplied dopamine (Ferkey et al., 2007). The *rgs-3* mutants tested off food respond more slowly to octanol than wild type animals; however, when tested on food *rgs-3* mutants respond more quickly. The rgs-*3; dop-3* double mutants do not revert to the wild type response time when on food, suggesting that the DOP-3 receptor is involved in modulating the response

of ASH. Expression of the wild type *dop-3* gene in the ASH neurons of *rgs-3; dop-3* double mutants is sufficient to rescue the wild type response to octanol when on food, and ASH-specific RNAi knockdown of *dop-3* recapitulated the *dop-3* genetic mutation (Ezak and Ferkey, 2010). However, *dop-3* expression in ASH neurons has not yet been demonstrated.

These studies indicate that dopamine signaling is important for the worm's ability to integrate information on repellant olfactory stimuli from the ASH neurons as well as information on food availability by dopaminergic mechanosensory neurons such as the CEP and ADE dopaminergic neurons. In this way, the worm can decide using both sets of information whether it is prudent to stay in the same area or move to an area with less potentially harmful chemicals.

1.3 Experimental Approaches to *Caenorhabditis elegans* Neurobiology

1.31 Microfluidics Allows for the Visualization of Neuronal Activity

Microfluidics is a powerful tool that allows for the visualization of neuronal activity in *C. elegans.* Microfluidic devices are made of the transparent silicone polymer polydimethylsiloxane (PDMS). These devices can be fabricated with laminar flow channels with widths on the order of micrometers. These microfluidic chips are ideal for *C. elegans* researchers because they can be made an ideal size for trapping worms in the chip for imaging. The small channels also ensure

that any liquid moving through the chip exhibits laminar flow. Laminar flow describes the phenomena where two different parallel liquid streams do not mix. Laminar flow occurs when liquids move through small channels at low velocities As PDMS is transparent, a worm trapped in the microfluidics chip can be easily imaged using an inverted microscope equipped with a CCD camera. The sections below will discuss microfluidics studies in *C. elegans* used to determine neuronal activity in response to stimuli, as well as the fluorescent sensor proteins that are crucial to monitoring neuronal activity *in C. elegans.*

1.31 a. Calcium Indicators Facilitate In- Vivo Visualization of Neuronal Activity. In order to directly observe neural activity within a neuron, a fluorescent indicator is often used. In C. elegans, Ca^{2+} indicators are often used because the influx of Ca^{2+} ions provide the main depolarizing current in the neurons of nematodes (Goodman et al., 1998) There have been a number of different Ca^{2+} sensors produced; for example, the aforementioned cameleon is an indicator that uses fluorescent resonance energy transfer, or FRET, to measure changes in intracellular Ca^{2+} levels (Llopis et al,,1997). However, some Ca^{2+} indicators that utilize FRET have been found to be somewhat insensitive to small changes in Ca^{2+} concentrations, which could result in loss of resolution (Truong et al., 2001). Therefore, other Ca^{2+} indicators have been produced that use the same principle of CaM and MLCKp interactions inducing fluorescence to measure changes in Ca^{2+} concentrations but are more sensitive to small Ca^{2+} fluctuations.

Once such molecule is known as GCaMP; this molecule utilizes the fluorescence of a circularly permutated enhanced green fluorescent protein (cpEGFP). The EGFP utilized in the protein GCaMP has had its N and C termini fused together; the middle of the protein is then cut such that these new termini are fused to either the CaM or M13 peptides. When no Ca^{2+} is bound to the cpEGFP, there will be little to no fluorescence due to a hole in the beta barrel of the cpEGFP. However, upon Ca^{2+} binding the CaM and M13 peptides will interact. This interaction results in a confrontational change such that the beta barrel is closed, and this change results in higher levels of fluorescence (Nakai et al., 2001) (Figure 5).

These Ca^{2+} sensors are very useful for in vivo measurements of neural activity not only because of their ability to fluoresce upon addition of Ca^{2+}, but also because they can be expressed in a cell specific manner. For instance, transgenic GCaMP can be specifically expressed in either the ASH sensory neuron or in the command interneurons by using cell specific promoter (Guo et al., 2009). This targeted expression ensures that the activity of your neurons of interest is being imaged. The plasmids containing genes coding for these Ca^{2+} sensors are typically introduced to the animal via microinjection into the syncytial gonad, which allows for the formation of an extrachromosomal array that is passed through the germ line (Mello et al., 1991).

1.31 b. Previous *Caenorhabditis elegans* Studies Utilizing Microfluidics Chips. Microfluidics is now widely used for a number of biological applications. Its main contribution to biology is that it greatly decreases the amount of time needed to perform various analyses via automation and micro scale processes. For example, microfluidics is used to facilitate DNA sequencing at the incredibly fast rate of 1.7 kb per minute. This was done utilizing a microfluidics chip containing 96 channels in which DNA strands were separated and analyzed to determine their sequence (Medintz, 2001). A microfluidics chip has also been produced that contains chambers in which PCR of a single DNA molecule is performed. These chambers are then connected to capillary electrophoresis channels. In this way DNA amplification and separation is coupled (Lagally et al., 2001). Microfluidics can also be utilized for single cell proteomics, which allows for the analysis of protein expression in single cells. To do this a single cell was taken from a cell reservoir within the chip and brought to a region in which a pulsed electric field was applied in order to lyse the cell. The contents of that cell were then labeled, separated, and quantified (Huang et al., 2007). Microfluidics also allows for the automation of enzymatic assays. A microfluidics device was created in which the researchers need only load the enzymatic solution and samples. These solutions would then be aliquotted, mixed, and analyzed in minutes with no further input from the researcher (Urbanski et al., 2008).

Microfluidics is not only useful for analysis on the level of a single cell; it can also be used to perform experiments on a whole organism such as *C.*

elegans. Because *C. elegans* is transparent it can easily be imaged using a PDMS microfluidics chip, which is also transparent. Microfluidics chips have been created that allow for the gene expression-based sorting of animals. In this approach a number of worms may be loaded into a single ship and each will be analyzed for GFP expression. If the animal is found to express GFP it will be routed towards the collection chamber in which the worm can be removed (Yanik & Rohde, 2007). Below are two examples of microfluidics being utilized to assess neuronal activity in *C. elegans* as well as responses to drugs, which illustrate the importance of microfluidics in this kind of experimentation.

One such study investigated a pair of olfactory neurons called AWC. These neurons are involved in directing chemotaxis of *C. elegans* to attractive odors as well as increasing turning probability during food searching. These neurons synapse onto the command interneurons AIY and AIB, which are involved in enhancing or suppressing turning, respectively. Although the synaptic connectivity of these neurons had been known for some time it was yet unknown how exactly odors affected their activity. To determine this, researchers utilized microfluidics, as well as the calcium sensor G-CaMP, to characterize the action of the AWC as well as the aforementioned command interneurons. AWC neurons were activated at odor removal. The longer the worm was pre-exposed to the odor the longer the response of the AWC neurons persisted. Furthermore, AWC integrates odor history over a period of at least 20 seconds, as shown by a

response of smaller magnitude when the animal was re-stimulated 20 seconds after the odor was first added.

When examining the command interneurons AIY and AIB it was discovered that AIB is activated by AWC upon odor removal and inhibited at odor addition. Similar to AWC, it was shown that AIB also has an increase in amplitude of the Ca^{2+} response when pre-exposed for longer amounts of time. The receptors involved in AWC-mediated activation of AIB are AMPA type glutamate receptors. AIY showed the opposite response; upon odor addition the neuron would become activated and upon odor removal it would be inhibited. Furthermore, when the AWC neurons were ablated the AIY response was completely eliminated. These results indicate that AWC inhibits AIY, even when AWC is at rest (Chalasani et al., 2007a).

The model of AWC response bears a striking resemblance to the ON and OFF bipolar cells of the vertebrate rod and cone receptors of the retina. These receptors are activated by the removal of light and are tonically active in the dark (Zhang and Cote, 2005). The neurons containing these receptors then synapse onto ON bipolar and OFF bipolar cells (Yang, 2004). Much the same way as AWC, photoreceptors are inhibited by the stimulus that they are produced to sense. This inhibition allows for the activation of ON bipolar cells. Upon light removal, however, the photoreceptors are activated and this inhibits the ON bipolar cells while activating the OFF bipolar cells (Figure 6). This study on AWC neurons in *C. elegans* is important, not only because it illustrates the power of

using microfluidics devices in *C. elegans* neurobiology, but it also shows that similarities can be found in the cellular and molecular mechanisms of the human and *C.elegans* nervous system.

Another such study looked at the gustatory neuron ASER response to NaCl in the presence and absence of ethanol. It has been known for some time that the ASE neuron senses the water soluble attractant NaCl but it was not known what effect a drug such as ethanol would have on the ability of ASER to sense changes in NaCl concentration. The ASE neurons, similar to the above mentioned AWC, are a bilateral pair of sensory neurons. In order to measure ASER activity, a microfluidics chip was used as well as the fluorescent Ca^{2+} indicator GCaMP; the activity of the neuron was measured as the change in fluorescence upon stimulation. The ASER neuron is known to exhibit an increase in activity when NaCl concentration decreases. This phenomenon is known as the OFF response. Conversely, the ON response describes the decreased activity of the ASER neuron upon a stepwise increase in the NaCl concentration. Upon exposure to ethanol it was established that while the OFF response was unaffected, the ON response was suppressed. When the animal was exposed to ethanol as well as increasing NaCl concentration, the activity of the ASER was reduced as compared to control animals.

The researchers also studied the effects of ethanol on sensory adaptation of the ASER neuron to continual stimulation of NaCl. When the animal was exposed to a steadily increasing concentration of NaCl for a period of 200

seconds it was noted that the activity of the ASER neuron would steadily decrease as the concentration increased. This same pattern was seen when the animal was exposed to ethanol as well as a steady increase in NaCl concentration; however, the decrease in activity of the ASER neuron was greater when the animal was exposed to ethanol. This suggested that ethanol contributes to neural adaptation upon continual exposure to a stimulus (Wang et al., 2011).

These studies exemplify not only the functional similarities among *C. elegans* and vertebrates such as mammals that make *C. elegans* a particularly viable model organism for neurobiology research, but also how powerful a tool microfluidics can be. In the past methods such as laser ablation and gene knockouts made it possible to see which neurons were involved in the behavior associated with particular stimuli. However, it was difficult to determine exactly how the neurons were able to cause this change in behavior. Now microfluidics chips, together with Ca^{2+} imaging, allow for conceptually straightforward visualization of neuronal activity of all the neurons in a sensorimotor circuit. In this way much can be learned about the physiological changes that result in behavioral changes in animals. Microfluidics also allows for very controlled exposure to both a stimulus and/or drugs.

As can be seen from the results above microfluidics enables the researcher to look at the actual neuronal activity that results in the behavior that is tested in various behavioral assays. The researchers above were able to not

only determine the neurons involved in a behavior, but in some cases the latency between exposure to stimulus and response as well as the effect of other chemicals on the response. I will do similar research in which I will utilize microfluidics to determine dopamine's effect on *C.elegans* response to glycerol, a high osmolarity stimulus. I will then be able to correlate the neuronal activity with the behavior tested in behavioral assays.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animal Husbandry

Animals were grown according to standard protocols as stated in Sulston et al 1974 (Sulston, 1974). Briefly, worms were grown at 15-25^0 C on 60 mm NGM plates seeded with 100 µL of a LB liquid culture of *E. coli* strain OP50.

2.2 Strain Construction

2.21 Breeding Schema

In order to produce a new strain ZY44 that contains both the *cat-2 (e1112)* mutation and the array driving GCaMP expression in ASH, the previously described strain CX10979 was utilized. This strain contains the *kyEx2865* array [*sra-6p::GCaMP;unc-122p::gfp*] in a wild type background. First, N2 animals were heat shocked at 30^{0}C for a period of 4 hours to increase male frequency and to establish a male stock (Sulston et al, 1988). N2 males were then mated with CX10979 worms. Fluorescent F1 males were then picked and allowed to mate to *cat-2* animals at 25^{0}C for 3 days; all resulting F2 fluorescent hermaphrodites are by necessity *cat-2* heterozygotes. Fluorescent F2 hermaphrodites were picked onto new plates and allowed to produce F3 self progeny; 15 fluorescent F3

animals were singled and allowed to drop eggs for one day. The parent F3 worm was then genotyped as described below to screen for animals homozygous for *cat-2 (e1112)*.

2.22 Single Worm Lysates

Single worm lysates were prepared by picking a single worm into a 200 µL PCR strip tube (Eppendorf, 1402-2700) containing 5 □L of worm lysis buffer (50mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, .045% Tween-20, 0.01% Gelatin). The worms were then flash frozen in LN_2 for two seconds. 5 □l of worm lysis buffer containing proteinase K (12/1000 vol. of 10mg/ml stock) was then added. Finally the tubes were incubated at 60^0C for one hour and subsequently at 95^0 for 15 minutes in a thermocycler.

2.23 Whole Plate Lysates

Whole plate lysates were performed to confirm the genotype of the worm strain after performing the single worm lysate. A plate full of worms is washed with MPGC (3.0g Na_2HPO_4, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.5 NH_4Cl, 1.0g PEG8000, 1.6 ml 5mg/ml cholesterol stock solution in 100% EtOH), this solution is then transferred into a 1.5 ml microcentrifuge tube and the worms are spun down using a benchtop picofuge. Some of the liquid is then removed until there is approximately 50-150 uL of solution left in the tube, 60 uL is then transferred to a new tube and placed in the LN2 for approximately 10 seconds. After removal, the tube is thawed and 120 □L of worm lysis buffer along with 4 □L of 10mg/ml

proteinase K is added. The tube is then incubated for 60 ^{0}C for one hour and 95 ^{0}C for 30 minutes.

2.24 Polymerase Chain Reaction (PCR)

For *cat-2* (*e1112*) genotyping, the PCR reactions were done utilizing the cat-2 #3 primer (GAGACATCTGAGCTAGCAGTGG) and cat-2 #4 (GCGAATGACGTCACTCCTATCG). The PCR conditions are as follows: for every one reaction 2 uL DNA lysate,3 uL 10x PCR buffer, 0.3 □L 25mM dNTP mix, 0.5 □L of both the cat-2 #3 and the cat-2 #4 primers, 0.3 □L of 25mM $MgCl_2$, 0.3 □L Taq polymerase, and 21.6 □L of water was added. The thermocycler was set to run for 2 minutes at 95 ^{0}C, 35 cycles of 95 ^{0}C for 30 seconds, 55 ^{0}C for 30 seconds, and 72 ^{0}C for 1 minute, and finally 72^{0}C for ten minutes to polish the DNA ends.

2.25 Restriction Digests and Gel Electrophoresis

For cat-2 (*e1112*) genotyping, the PCR product was digested with restriction enzyme *Dde*I (Fisher Scientific) for 2 hours at 37^0 C. The restriction products were then separated on a SFR agarose gel (Bio-express, 9012-36-6) according to the vendor's instructions to determine the genotype of the worms. Gel electrophoresis was performed in a gel apparatus (CBS Scientific) equipped with a peristaltic pump to circulate the electrophoresis buffer (1x TAE) as well as a separate cooling chamber attached to a circulating cooler (Fisher Scientific) set at 4^0 C.

2.3 Drop Assay

Drop assays were performed based on a modified protocol of a previously described method (Hilliard et al., 2002). Four NGM plates are used per experiment with five worms placed on each plate. These worms are allowed to equilibrate for a period of ten minutes before exposure to stimulus. The worms are then stimulated by applying a drop of the stimulus near the tail of the worm using a drawn out glass microcapillary pipette (1. MM,VWR International,TW100F-4). This drop moves up the worm via capillary action until it reaches the worm's amphid pore, where the stimulus will be sensed. The response time, or the time for the worm to reverse direction, was then recorded. Every worm on each of the four plates was tested four times such that every plate represents twenty individual drops. From this data an avoidance index was calculated. The avoidance index is defined as the ratio of drops that elicited a response within two seconds compared to total number of drops.

2.4 Chip Protocol

2.41 Producing the Microfluidics Chip

An AutoCad file containing the negative image of the previously described olfactory chip (Chronis et al., 2007) was kindly provided by Dr. S. Chalasani (Salk Institute). This design was sent to the Microfluidics Foundry at Stanford

University for mask production. This mask was then used to cast the microfluidics chip utilizing Sylgard 184 silicone elastomer (Ellsworth, cat. no. 4019862). To produce the silicone polymer, 6.4 g of the silicone elastomer curing agent is added to 64 g of the silicone elastomer base; this solution is then manually mixed for a period of fifteen minutes using a 1000 µL pipette tip. The solution is then poured over the mask in a 150 mm Petri dish and placed in a vacuum desiccator (Fisher Scientific, cat. no. 420250000) for a period of 90-120 minutes. The chips are then cut away from the mold using a sharp scalpel blade. The holes for each fluid inlet port are punched utilizing a Harris Uni-Core I.D. 0.75 mm (Sigma Aldrich, cat. no. Z708798). The chips are then bonded to 24 x 50 mm cover glass (Fisher Scientific, cat. no. 12-544 C) utilizing a hand held corona generator (Electro-Technic Products, SKU #: 12051A-10) for 2 minutes. The chips are then bonded to the cover glass overnight. These chips are then ready for use.

2.42 Assembly of the Recording Rig

Herein I describe in detail the procedure to establish fluid flow in the microfluidics chip. Each solution is prepared in M13 buffer (30 mM Tris, 100 mM NaCl, 10mM KCl). In order to interface the vacuum with the chip, the vacuum source is first connected to vacuum tubing with an inner diameter of 1/4 inch. A male Luer lock to large 1/4 in. barb adaptor is used to connect a disposable dispensing needle to the vacuum tubing (McMaster-Carr, cat. no. 75165A684). Flexible Tygon tubing (ID 0.02 in., O.D. 0.06 in.) is then attached to this needle; this is the tubing that will eventually be inserted into the inlet ports of the chip.

The Tygon tubing must then be connected to a piece of metal tubing (New England Small Tubing,I.D. 0.017 in. O.D. 0.025 in.), the other end of which is inserted into the vacuum inlet port of the chip. The rest of the inlet ports used for fluid flow are set up in a similar fashion; however, the Tygon tubing is instead connected to 50 c.c. syringes serving as fluid reservoirs (Fisher Scientific, cat. no. 14-817-33). The tubing is connected to the aforementioned Luer lock dispensing needles, which are then connected to two-way stopcocks (Bio-Rad, cat. no. 732-8102). These stopcocks are then connected to the fluid reservoirs. Although there are five fluid inlet ports on the chip, only four fluid reservoirs are needed. This is because the two dye inlet ports are supplied by the same fluid reservoir. The dye reservoir is connected to a Lee 12V three-way solenoid valve (Warner Instruments, cat. no. 64-0175 (SV-1)). This valve ensures that only one of the two dye channels has fluid flow at any one time. Valve control was initially prototyped using a breadboard design and standard electronic components (Figure 7). Once chip assembly became more routine, a commercial valve control system (Warner Instruments, VC-66MCS) was used for more robust switching and control. The reservoir connecting to the worm inlet port is connected to the tubing via a three way stopcock (Cook Medical, cat. no. G14123) rather than a two-way stopcock. In addition to interfacing the reservoir and the tubing, the third connector is connected to a 3 c.c. syringe (Fisher Scientific, cat. no. 14-829-13C) filled with M13 buffer.

2.43 Recording Calcium Transients Within a Microfluidics Chip

To record calcium transients within a microfluidics chip worms were first loaded into the chip. To insert an animal into the chip, place an adult *C. elegans* hermaphrodite onto a drop of M13 on a clean NGM plate. The tubing that supplies the worm inlet port is disconnected from the chip, and the three way stopcock that supplies the worm inlet port is switched to the syringe. The syringe is used to generate a gentle suction such that the worm is taken up into the tubing. The tubing is then re-connected to the chip. The worm should be pulled through the worm inlet port and into the chip, which can be visually tracked using the stereomicroscope. Once the worm has entered the chip the syringe plunger is used to push the worm further into the trap until the nose of the animal is just sticking out of the worm trap. Once the chip is set up, the entire apparatus is transferred to the stage of an inverted fluorescence microscope.The worms were then allowed to acclimate to the chip for a period of two minutes. The worm was then exposed to blue light for two more minutes. This was done because ASH is known to respond to blue light, and that pre-exposure to blue light for one to two minutes is sufficient to eliminate this response (Hilliard et al., 2005). After pre-exposure the recordings began. For ten seconds the worms were recorded without being exposed to stimulus, which allowed for the determination of baseline fluorescence. The worms were then exposed to the stimulus for a period of 10-15 seconds, after which time the recording remained on for 10 seconds. All ASH microfluidics experiments were performed with the CX10979 and ZY44

strain described above. The pharyngeal recordings were done with the SGLO strain and .203 f of serotonin in 20 ml of M13.

The recordings were taken using a Retiga SRV cooled CCD camera attached to a Nikon TU-2000 inverted microscope with an X-Cite transluminator. An imaging workstation (Lenovo workstation with Intel i7-2600 CPU @3.40 GHz and 16 GB RAM running Windows 7 Professional 64 bit) with Micro Manager 1.4.14 (http://valelab.ucsf.edu/~MM/MMwiki/.) was used to capture videos (0.817 gain, 0 offset, 20MHz readout time, and 90 ms frame rate). The videos were then analyzed with Image J (NIH, v1.43m). Statistical analysis was done in Microsoft Excel utilizing the data analysis pack.

CHAPTER THREE

RESULTS

3.1 Aversive Response to the Soluble Repellants Glycerol and SDS is Modulated by Dopamine

Previous studies in our lab (M. Genovez and M. Baidya, unpublished) and others had shown that ASH mediated aversive response to 1-octanol is modulated by dopamine. This was shown by performing behavioral assays in which a forward moving worm is exposed to 100% octanol and the time to initiate a reversal is documented. Wild type worms were found to respond within approx. 2 seconds while *cat-2* mutant responded within approx. 4 seconds. Rescue experiments were performed in which the worms were tested in the presence of exogenous dopamine and then tested for avoidance response. Exogenous dopamine was found to be sufficient to restore normal response to the *cat-2* mutant worms. (Figure 8). Thus, ASH mediated avoidance responses are likely modulated by dopamine.

To determine the role of dopamine in modulating ASH neuronal activity, I utilized optical recording in the microfluidics chip. However, octanol cannot be used in a microfluidics chip because it is a hydrophobic organic compound and therefore not soluble in water. In addition to volatile compounds, ASH also mediates response to several soluble compounds, including quinine, SDS, glycerol and Cu^{2+} (Hilliard et al., 2004c). We therefore determined if *C. elegans* response to any of these compounds is modulated by dopamine in a similar way.

We first tested quinine at three different concentrations (Figure 9). Worms are typically stimulated with soluble tastants using the previously described drop assay. At all three concentrations tested, wild type and *cat-2* mutant animals responded equally well to quinine. Interestingly, there was no dose dependence in the response.

We next tested the soluble repellant SDS. The *cat-2* mutant animals responded significantly worse to 0.1% SDS (prepared in M13 buffer) than wild type (Figure 10). To determine if the defective response in *cat-2* mutants is due to a deficit in dopamine levels, rescue experiments with exogenous dopamine were performed. Exogenous dopamine was directly added to NGM plates a few minutes before placing worms onto plates for testing. Treatment with dopamine increased the number of responders for both wild type and *cat-2* mutant animals. Treatment of animals with vehicle only (H_2O) had no effect. Interestingly, these results suggest that the levels of endogenous dopamine in wild type animals under our assay conditions are less than what is needed to exert its full effect on aversive behavior.

Glycerol, which stimulates the avoidance response to high osmolarity mediated by ASH neurons (Hilliard et al., 2004a), was also tested utilizing the drop assay (Figure 11). Similar to SDS, the avoidance response to 1 M glycerol (prepared in M13 buffer) is modulated by dopamine. Unlike SDS, however, *cat-2* dopamine deficient mutants responded better than the wild type animals. Upon exposure to exogenous dopamine the *cat-2* worms response reverted to wild

type levels. A vehicle control in which the worms were exposed to only water was also performed and found to have no effect on response.

These experiments indicate that dopamine modulates the avoidance behavior of *C. elegans* to SDS and glycerol but probably not quinine. In retrospect this is not too surprising as loss of function in the *qui-1* gene affects *C. elegans* response to quinine but not SDS (Hilliard et al., 2004c), suggesting that these two tastants are detected by distinct signaling pathways. Therefore, glycerol was chosen as the stimulant of choice in the microfluidics recordings (see Experiments below).

3.2 Chip Protocol

3.21 The Olfactory Chip

To optically record neuronal activity from ASH neurons, I utilized a modified version of the previously described olfactory chip (Chronis et al., 2007). The chip design is shown in Figure 12. The olfactory chip contains 4 fluid inlet ports, 1 worm inlet port, and 1 vacuum port. The four inlet ports from left to right are: the first dye channel, the stimulus channel, the buffer channel, and the second dye channel. At any one time only one of the dye channels will have fluid flow. When the first dye channel has fluid flow the buffer solution will be diverted towards the worm inlet port such that the nose of the worm is only exposed to

buffer. However, when the second dye channel has fluid flow the stimulus channel will be diverted towards the worm inlet port such that the worm is exposed to the stimulus. The design utilized in this study includes an additional channel that switches between buffer and exogenous agonist, which in this study is dopamine (see below).

3.22 Making an Olfactory Microfluidics Chip

An AutoCad design containing the negative image of the chip is needed to generate a mask for casting a microfluidics chip using the silicone polymer PDMS (Figure 13). These masks can be obtained from a number of different laboratories (e.g., Stanford Microfluidics Foundry, Stanford Univ.) that specialize in microfluidics chip and mask production. Typically, staff members of these facilities will ensure that the AutoCad files contain no errors and are in an appropriate format. The masks are produced by first printing a high resolution transparency of the AutoCad file. This transparency is then placed onto a silicone wafer which has been covered in a photoresist solution. The wafer is then exposed to UV light which activates any of the photoresist that is not covered by the transparency. The activated photoresist breaks down the silicone polymer such that channels are produced. The photoresist is then washed away revealing the pattern of the silicone mold (Duffy et al., 1998).

Masks are placed in a large Petri dish in a clean environment (e.g., a laminar flow hood), and PDMS reagent is prepared and poured into the dish (see Materials and Methods for details). Once the PDMS has fully polymerized, the

chips are cut away from each other and excess PDMS using a scalpel. Each casting produces 20 chips. Each individual chip must now have 0.75 mm holes punched at each of the six fluid inlet ports; these ports will eventually be used to insert the tubing that will supply fluid flow to the chip. The holes are punched under a dissecting stereomicroscope using a Harris Uni-Core tool (Figure 14). Prior to bonding, surface contaminants such as dust particles are removed using Scotch tape.

The chips are now ready to be bonded to a 24x50 mm cover glass. Previously published methods for the *C. elegans* olfactory chip utilized a plasma oven for charging the surfaces of PDMS and cover glass. I utilized a modified method recommended by the Stanford Microfluidics Foundry (Haubert et al., 2006). The chip and glass are electrically charged on their surface using an Electro-Technic, Inc. Model BD-20 handheld corona generator placed 0.635 cm above the surfaces to be treated. The chip is treated for a period of two minutes, and subsequently the glass is then treated for two minutes as well. Once both the chip and the glass have been treated they are bonded together by simply placing the chip on the cover glass. The chips are then allowed to sit overnight in order to ensure complete bonding. Overnight bonding is absolutely critical, because insufficient bonding time results in poorly sealed chips, which will result in leakage when establishing fluid flow (see below).

3.23 Setting Up Fluid Flow Through the Chip

Three separate solutions are flowed through the olfactory chip. First, M13 buffer is used for both the buffer inlet port and worm inlet port. An agonist or antagonist solution can be diverted to this buffer channel using a solenoid valve (see below). Second, 0.05 mM fluorescein dye solution in M13 is used in the dye port and is utilized to visualize fluid flow during fluorescent imaging. Third, the stimulating reagent (e.g., SDS) in M13 buffer is used in the stimulus port. Prior studies with the olfactory chip utilized S basal as the solvent; however, as K^+ ions in S basal cause SDS to precipitate, M13 buffer was used instead, which has a lower K^+ concentration. All solutions must be filtered to remove any particulates that might block flow through the chip.

Once the tubing has been properly set up, they can now be inserted to each of the inlet ports. The order in which the tubing is added to each inlet port is important in establishing laminar fluid flow. First, the vacuum supply must be connected to the vacuum inlet port found at the very bottom of the chip and vacuum applied at approx. -0.7 BAR for a period of thirty minutes. Second, the worm inlet port right above the vacuum port is connected. Vacuum within the chip and gravitational pull should now establish liquid flow in this channel (and subsequent channels as well), which can be visually confirmed using a stereomicroscope. Third, the buffer ports are connected. Fourth, the two dye ports are connected. Ensure that the solenoid valve connected to the two dye channels is correctly switched such that the outflow port connects to the chip.

The dye inlet port next to buffer inlet port is connected first and the dye inlet port next to the stimulus inlet port is next. Finally, the stimulus inlet port is connected. When connecting each of the fluid channels to the chip it is important to make sure that each solution is running smoothly through the chip before the next solution is added. If proper flow is not established at any step, the apparatus must be disassembled and the entire procedure should be repeated from the beginning. Figure 15 shows the chip with all tubing interfaced.

To insert worms into the chip the tubing that supplies the worm inlet port is removed and the stop cock attached to said tubing is switched such that the fluid originates from the syringe. The syringe is used to suck a worm into the tubing from a NGM plate. The tubing is then re-inserted into the chip and the light pressure is applied to the plunger in order to push the worm into the worm trap. Once the chip is set up, the entire apparatus is transferred to the stage of an inverted fluorescence microscope. Figure 16 shows the complete rig setup.

3.3 Construction of a Transgenic Strain for Calcium Recordings

Animals that were homozygous for the *cat-2 (e1112)* mutation and carrying the array (*kyEx2865*) driving GCaMP expression in the ASH neuron were generated (see Materials and Methods). The *e1112* allele is a single nucleotide polymorphism (SNP) that generates a *Dde*I restriction fragment length polymorphism (RFLP). Thus, a *Dde*I digest of a PCR product generated with

primers flanking this SNP can be used to genotype progeny from a strain construction cross. Figure 17 shows that F3 progeny with the desired *cat-2 (e1112); kyEx2865* genotypes were obtained. This new strain was named ZY44, and was utilized to test the effect of dopamine on ASH activity upon exposure to glycerol within the microfluidics chip.

3.4 Measuring Fluorescence in the Pharynx and Prototyping the Drug Delivery Rig

In order to ensure that Ca^{2+} transients could be recorded within the olfactory chip, a transgenic strain (SGlo) that expresses GCaMP in its pharyngeal muscles was utilized (a gift from S. Lockery, U. Oregon). Much like neuronal activity, muscle activity can be assessed by monitoring Ca^{2+}transients. Initial experiments revealed that when *C. elegans* is inserted into the worm trap in the chip, the pharynx stops pumping, presumably due to a lack of. To stimulate pumping, the animals were exposed to 25 mM serotonin to induce pharyngeal pumping. Serotonin is known to cause rapid contraction and relaxation cycles of the pharyngeal muscles (Niacaris, 2003). This experiment also had a side benefit; it was also used to prototype the drug delivery system.

The drug delivery rig was designed such that an extra fluid reservoir containing the serotonin solution was connected to a second solenoid valve along with the reservoir containing the buffer solution. This solenoid valve was

then connected to the buffer channel of the olfactory chip. Upon activation of the solenoid valve the buffer solution would be replaced with the serotonin solution, which would then flow through the chip past the nose of the worm.

I first determined the flow rate of the solution, so that the animal's time of exposure to the drug could be calculated. To do this, a placebo solution containing 0.05 mM fluorescein was loaded into the fluid reservoir, and the time for fluorescent signal at the chip to be observed once the solenoid valve was activated was measured. Since flow rate depends on vacuum pull and gravitational pull, the height of the fluid reservoirs (as controlled by their placement on the ring stand) and the vacuum must be set consistently. When the solenoid is switched off, the time of drug clearance depends on the null volume of the tubing. Under the conditions used for the preliminary studies described herein, the time for the drug solution to reach the worm was approximately 52 +/- 3.05 seconds (n=5) and the time for it to be cleared was approximately 2 minutes 30 seconds +/- 7.26 seconds (n=5). These parameters must be empirically derived whenever the rig is reassembled with new tubing.

In the absence of serotonin, SGlo worms pumped at a basal rate of roughly 5 pumps/minute, although pumping was erratic and difficult to quantify. Upon exposure to 25 mM serotonin, the rate of pumping drastically increased to 200 pumps per minute. Pumping began approximately 1 minute 43 seconds after the solenoid was activated, suggesting that a sufficient amount of serotonin had diffused laterally from the buffer stream. It also suggested that the

pharmacokinetics of serotonin is quite rapid, as pumping began approximately 50 seconds after serotonin reached the worm's nose.

Videos of the pumping pharynxes of SGlo worms were captured. The recordings were analyzed using NIH ImageJ, and pixel intensity was measured and plotted as percent increase of fluorescence above baseline as a function of time. Figure 18 shows a characteristic trace of a contracting pharynx; high pixel intensity is indicative of increased Ca^{2+} concentration within the cell. The frequency of Ca^{2+} spikes (888+/-30 spikes/min., n=3) exceeds that of pharyngeal pumping rates (126+/-37.6 pumps/min., n=5), as assessed by recordings performed in the visible light channel, suggesting that there is some kind of threshold of Ca^{2+} levels that triggers pharyngeal pumping. These results indicate that: (1) the recording rig is capable of recording Ca^{2+} transients with good temporal and spatial resolution, and that (2) the drug delivery rig is capable of delivering a soluble agonist to the animal, and that such an agonist can diffuse laterally into the animal with relative rapid kinetics. With these results, recording dopamine-modulated Ca^{2+} transients within the ASH neuron is now technically feasible.

3.5 Measuring Calcium Transients in the ASH Neuron in Response to Stimulus

3.51 The ASH Neuron Shows No Response Upon Exposure to SDS

In order to determine the response of ASH to the detergent SDS worms were tested within a microfluidics chip as described above (see Materials and Methods). Figure 19 shows the average trace for the seven wild type worms tested. There was no obvious change in intracellular Ca^{2+} levels in ASH neurons in response to 0.1% SDS. This was somewhat surprising as there was a previous study that indicated that ASH was involved in sensing SDS. However, that same study also showed that another sensory neuron, ASK, had a more prominent role in the SDS avoidance response (Hilliard et al., 2002). It it possible that the ASK neurons and not the ASH neurons are the primary neurons involved in sensing SDS.

3.52 The ASH Neuron Responds Upon Exposure to Glycerol;This Response is Modulated by Dopamine

Glycerol was then tested to determine if it elicited a response from the ASH neuron. It had previously been shown that avoidance to glycerol was modulated by dopamine (Figure 10). However, whether this modulation occurred at the site of the ASH neuron was not yet established. Figure 20 shows the average calcium response of wild type (n=7) and *cat-2* mutant worms (n=11) in response to exposure to glycerol. At approximately 10 seconds the worm was exposed to 1M glycerol stimulus, and the stimulus was removed at approximately 20 seconds. The worms responded approximately 1 second after exposure of

stimulus in both the wild type and *cat-2* worms. Upon removal of the stimulus however, *cat-2* mutants exhibit a second spike in activity which is not as pronounced in the wild type worms. In order to analyze the videos ImageJ was utilized to measure pixel intensity of the ASH neuron in each frame of the recording. This data was then further analyzed to determine fluorescence above baseline. Any recording that had a 400% increase in fluorescence in two or more frames was not used for statistical analysis as 400% is over the dynamic range of GCaMP (Chalasani et al., 2007b). In order to compare the responses, the changes in fluorescence from frame 210-255 were compared between N2 and *cat-2* mutants. These frames correspond to the time interval one second before stimulus removal to three seconds after stimulus removal (a 4-second window). This time span was chosen as this was the time in which the strains differed the most. During this time the *cat-2* mutants were found to have a ~35% greater increase in fluorescence as compared to that of the wild type worms (p value = 5.35×10^{-9}, Student's t-test). Four other 4-second time intervals were also compared from frame 99 to frame 210, representing the interval during which fluorescence initially increased due to exposure to stimulus. The difference between strains was found to be statistically insignificant along this entire time interval ($p>0.05$). This indicates that the difference between the dopamine deficient mutants and the wild type is found only after stimulus is removed. This result also indicates that dopamine potentiates the response of the ASH neurons in response to high osmotic stimuli.

CHAPTER FOUR

DISCUSSION

Dopamine has been shown to be involved in many important processes within the human brain. These range from learning and cognition to disease phenotypes such as drug addiction and Parkinson's disease. In order to understand the molecular and cellular mechanisms underlying dopamine signal transduction a model organism is often utilized. The nematode *C. elegans* is an excellent model organism for these kinds of studies. Although their nervous system is simple they utilize dopamine at the molecular and cellular level in much the same way as mammals. This study looked at the effect of dopamine on the avoidance response of *C. elegans* to different aversive stimuli in an effort to elucidate the mechanism and site of dopamine modulation in these behavioral responses. This was done by performing behavioral assays as well as directly visualizing neuronal activity of the ASH sensory neurons.

Behavioral assays are useful for studying the effects of neurotransmitters on behavior, but it is difficult to determine the cellular site of action for these neurotransmitters utilizing just this method. With the advent of microfluidics chips and calcium sensitive fluorescent proteins, the activity of a single neuron can be imaged in response to stimulus. These two methods, used together, can give a better picture of the effect of the neurotransmitters on the behavior. Behavioral assays can be used as a preliminary approach to determine whether a

neurotransmitter affects a particular behavior. Once this is determined, one can then perform microfluidics studies to determine what neurons are being affected and result in this change in behavior. My experiments utilized this method to first determine what stimuli elicited a dopamine-modulated avoidance response. Once this was determined, microfluidics experiments were utilized to determine where this modulation might be occurring.

Although many aversive stimuli are sensed by the ASH sensory neurons it was unknown whether any of these behavioral responses were modulated by dopamine. Some earlier studies showed that dopamine plays a role in regulating ASH response to octanol, but in somewhat convoluted behavioral paradigms (Ezak and Ferkey, 2010). In order to more carefully address the role of dopamine in ASH mediated behaviors, drop assays were performed with three different stimuli on both wild type and dopamine deficient *cat-2* mutants. My data suggested that the behavioral response to SDS, glycerol (and to octanol; Baidya, Genovez, and Chao, unpublished data) is modulated by dopamine whereas response to quinine was not. This is biologically interesting as it suggests that different stimuli are differentially modulated through different pathways within the same neuron. The ASH neurons are polymodal sensory neurons that detect mechanical nose touch (Hart et al., 1999; Kaplan and Horvitz, 1993), high osmolarity (Hart et al., 1999) and volatile repellant chemicals (Troemel et al., 1999). Various mutants have been isolated that affect one but not other sensory modalities. For instance, *osm-10* codes for a novel cytoplasmic protein

expressed in ASH neurons and is required for response to glycerol but not other sensory modalities (Hart et al., 1999). Hilliard et. al, (2004) also showed that the Gα protein GPA -3 must be expressed in order to sense quinine, but is not needed for glycerol response. It has also been shown that response to SDS and quinine, but not other ASH detected stimuli, require the *qui-1* gene (Hilliard et al., 2004c). Other genes appear to be part of the core functionality of ASH neurons. For instance, *osm-9* and *ocr-2* code for TRP channels that are essential for all ASH sensory modalities (De Bono et al., 2002). The *eat-4* gene codes for a vesicular glutamate transporter that is required for glutamatergic neurotransmission at the synapse (Lee et al., 1999). Thus, it is possible that dopamine exerts its effects between modality-specific and core signaling components of ASH neurons.

It would be interesting to perform a variant of the drop assay in which the stimuli to the worm's head instead of its tail. This assay is performed by placing a drop of liquid on the agar in front of the worm. This solution is then allowed to soaks into the agar before the worm comes into contact with it so that a nose touch response is not elicited. The worm can still "taste" the stimuli and will react to it in much the same way as the normal drop assay. This assay differs from the normal drop assay as it removes the involvement of the phasmid sensory neurons found in the tail. This could possibly change the result for SDS as Hillard et al. (2002) showed that the phasmid neurons antagonize the avoidance response of *C. elegans* in response to SDS.

Behavioral tests such as the drop assay assess the activity of an intact neural circuit, which includes the sensory neurons, command interneurons, and motor neurons as a whole. The drop assay data therefore indicate that the response to both glycerol and SDS are modulated by dopamine but it gives no information about the site of modulation. In order to determine this, calcium sensitive fluorescent proteins such as GCaMP can be utilized to visualize activity within a single neuron. This is often done within a microfluidics chip which allows for precise control over stimulus exposure as well as a way to immobilize the worm for proper imaging. Microfluidics experiments were performed utilizing both glycerol and SDS, as they both showed dopamine modulation in the drop assays. All wild type worms that were tested showed no increased ASH activity upon exposure or removal of SDS as stimulants. This is surprising as Hilliard et al. (2004) showed that ASH is involved in sensing quinine. However, in that study it was noted that the avoidance response to quinine was most strongly affected when both ASH and another sensory neuron ASK were ablated. My data taken along with that of Hilliard at al. (2004) indicates that the response of ASK to SDS is more important to the avoidance response. It would be interesting to assess the response of ASH neurons to quinine in the context of ASK-ablated animals.

Glycerol has been previously shown to evoke calcium transients in ASH neurons (Chronis et al., 2007). Approximately a second after stimulus exposure the ASH neurons in wild type animals showed a ~200% increase in fluorescence (Figure 20), consistent with previously published data (Chronis et al., 2007). A

similar response was seen for both the wild type and dopamine deficient *cat-2* worms. However, the wild type and *cat-2* strains differed in their response to the removal of stimulus. The wild type strain showed a modest increase in fluorescence while the *cat-2* dopamine deficient mutants showed a 35% greater increase in activity when stimulus was removed. This suggests that the modulation of glycerol's response by dopamine may occur (at least partially) within the ASH neurons. This increased activity within the ASH neurons could explain why *cat-2* mutants responded better than wild type animals in the drop assay (Figure 10).. Dopamine could possibly modulate other neurons of the ASH avoidance circuit as well. For example, serotonin modulates the responses of multiple neurons within the ASH avoidance circuit (Harris et al., 2009). The next logical experiment would therefore be recording calcium activity of command interneurons as well as of motor neurons that are also a part of the avoidance circuit.

Identifying the receptors involved in dopamine modulation would also be an extension of this work, and would provide more conclusive evidence determining whether or not ASH is the direct site of dopamine modulation. The most obvious candidate receptor to test would be the DOP-3 receptor, which has been shown to be involved in the response to octanol and may function in the ASH neuron (Ezak and Ferkey, 2010). The involvement of DOP-3 could be tested by constructing animals that harbor a mutation in *dop-3* and express GCaMP in the ASH neurons, and recording the neural activity in response to

stimulus such as glycerol. Rescue experiments in which the missing dopamine receptor was transgenically expressed specifically in the ASH neurons would also be performed in order to show that the receptor was indeed involved in the modulation. This same procedure would need to be done for all dopamine receptors, as it is possible that multiple dopamine receptors are expressed on the ASH neurons.

It is also possible that other amines, such as tyramine or octopamine, modulate the ASH response. For instance, the octopamine receptor designated F14D12.6, is expressed in the ASH neurons and affects its response to octanol (Wragg et al., 2007). Tyramine and serotonin also potentiate activity in ASH neurons (Chao et al., 2004; Wragg et al., 2007). It would be interesting to determine whether these different modulatory neurotransmitters have modality-specific effects and whether they have synergistic and/or antagonistic effects in modulating ASH activity. These different neurotransmitters likely allow the worm to integrate multiple lines of information about their environment. For example, serotonin seems to be released in response to an animal finding food after having been food deprived. Upon entering food the worm will completely stop locomotion in order to ensure it stays within the food. This response is greater than the slowing response modulated by dopamine, which is seen in well fed worms. Utilizing two neurotransmitters to affect locomotion upon finding food allows for different responses depending on if the worm is in desperate need of that food (Sawin et al., 2000). Dopamine also allows for the integration of food

availability and response to vibrations. In the presence of food *C. elegans* habituates to repeated vibrations or taps slower than if off food. This behavior is known to be facilitated by dopamine signaling via the DOP-1 receptor. It is believed that this decreased habituation response allows for the worm to remain vigilant while in food, as predators will have a higher probability of being there as well (Kindt et al., 2007). Tyramine and octopamine are also preliminarily believed to be involved in signaling low food availability (Alkema et al., 2005; Horvitz et al., 1982; Rex et al., 2004), but more study is needed on the activity of these two neurotransmitters.

APPENDIX A

FIGURES

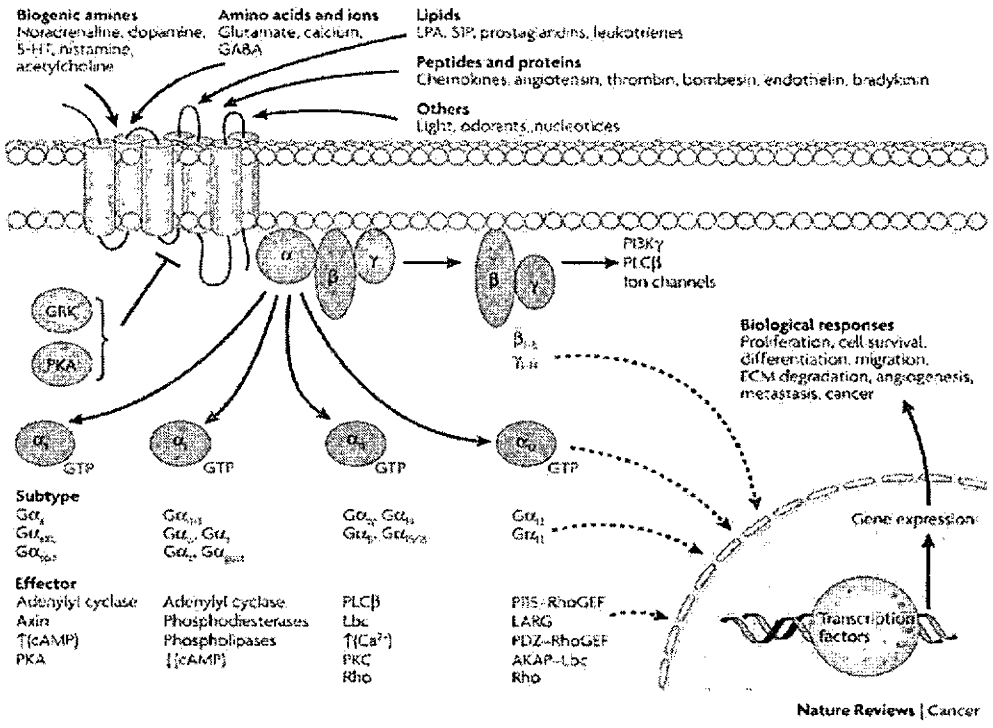

Figure 1. GPCR signaling cascades within a eukaryotic cell. General schematic of G protein coupled receptor (GPCR) and heterotrimeric G protein signaling. After ligand binding to the GPCR, the G protein, which is composed of alpha, beta, and gamma subunits, dissociates from the receptor powered by the hydrolysis of GTP bound to the alpha subunit. The beta and gamma subunit then dissociate from the alpha subunit and both go on to activate secondary messengers.

Dorsam, R., and Gutkind, J.S. (2007). G-protein-coupled receptors and cancer. Nature Reviews. Cancer *7*, 77–94.

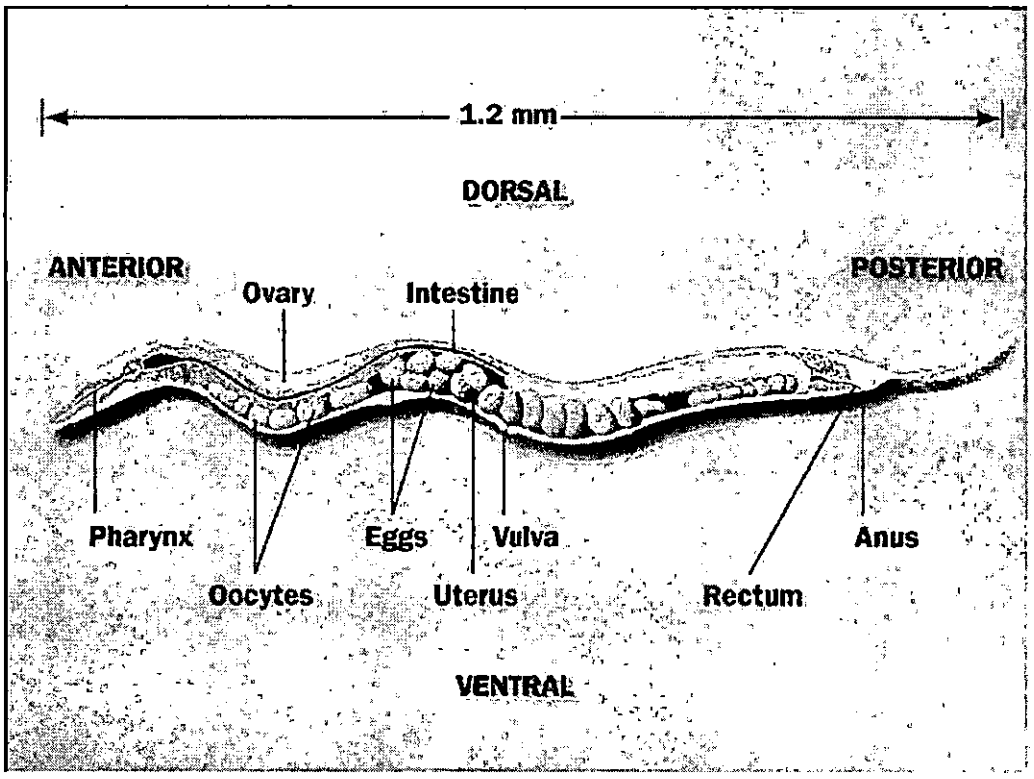

Figure 2. Gross anatomy of a *Caenorhabditis elegans* hermaphrodite. The adult animal is approx. 1 mm in length.

[Untitled schematic of a *C.elegans* hermaphrodite]. Retrieved February 13,2012, from:

http://www.imsc.res.in/~sitabhra/research/neural/celegans/index.html

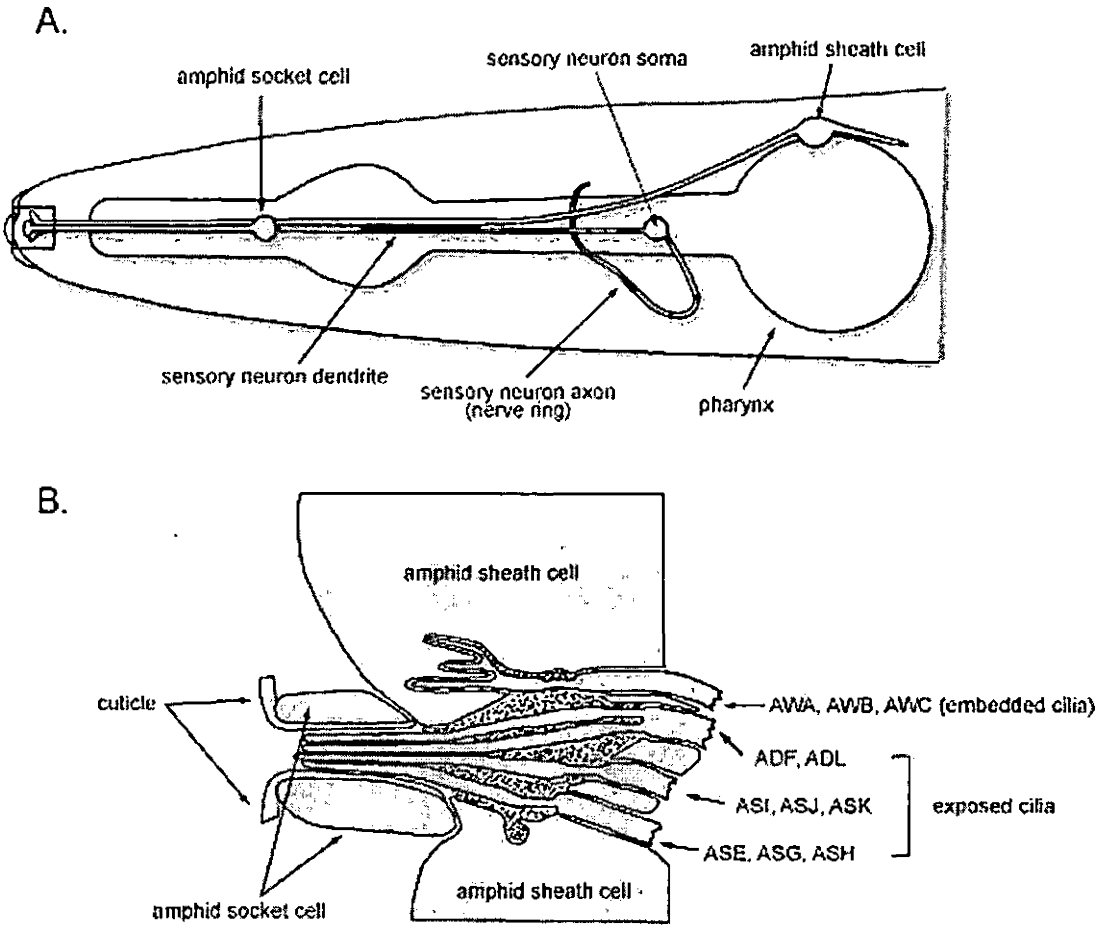

Figure 3. Neurons present within the amphid pore of *C.elegans.* The amphid pore is the anterior sensory organ of *C. elegans.* The amphid pore and the ciliated dendrites extending out of it are shown. Twelve neurons sense the environment via this pore. Of these twelve eight are directly exposed to the environment while four are indirectly exposed. This image © Michael Chao; released under the Creative Commons Attribution-Share Alike 3.0 License (see http://creativecommons.org/licenses/by-sa/3.0/us/ for details).

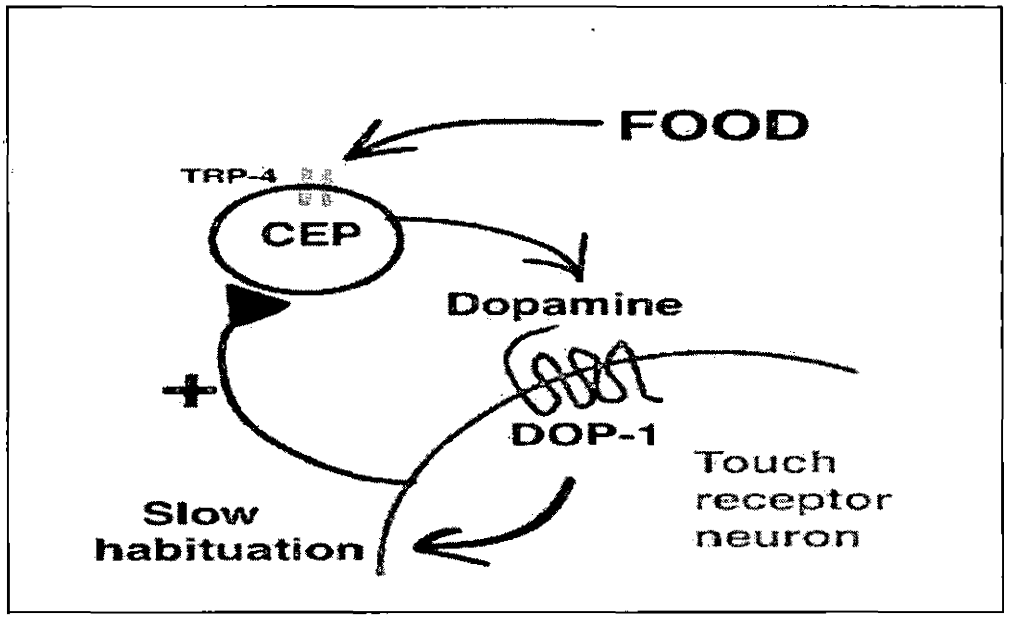

Figure 4. Dopamine modulates *C. elegans* response to touch. The presence of food causes the CEP dopaminergic neurons to release dopamine. Dopamine modulates tap habituation in the touch receptor neurons via the DOP-1 GPCR such that in the presence of food habituation will occur at a slower rate.

Kindt, K.S., Quast, K.B., Giles, A.C., De, S., Hendrey, D., Nicastro, I., Rankin, C.H., and Schafer, W.R. (2007). Dopamine mediates context-dependent modulation of sensory plasticity in C. elegans. Neuron *55*, 662–676.

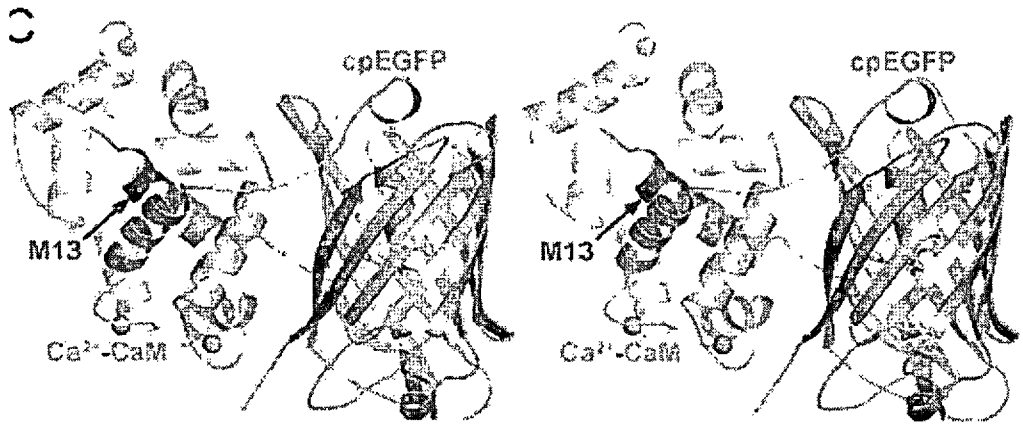

Figure 5. Structure of the Ca^{2+} sensing molecule GCaMP. The molecule is composed of an M13 myosin light chain, a calmodulin domain (CaM), and a cpEGFP molecule. Upon binding of Ca^{2+}, the calmodulin and myosin light chain domain interact which allows the GFP molecule to fluoresce. In this way Ca^{2+} levels can be quantified *in-vivo.*

Akerboom, J., Rivera, J.D.V., Guilbe, M.M.R., Malavé, E.C.A., Hernandez, H.H., Tian, L., Hires, S.A., Marvin, J.S., Looger, L.L., and Schreiter, E.R. (2009). Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. The Journal of Biological Chemistry *284*, 6455–6464.

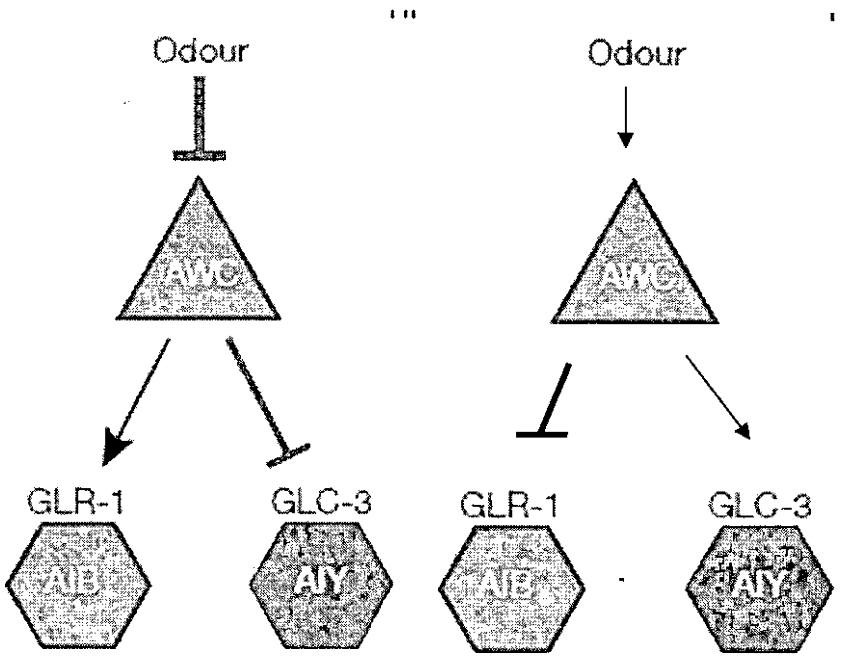

Figure 6. Model of the AWC, AIB, and AIY neural circuit. Upon removal of odor the AWC sensory neurons are inhibited, which allows for AIB activation. Upon odour presentation, however, AWC is activated as is AIY. These interneurons form complex synaptic connections with the AVA, AVB, AVD, AVE, and PVC command interneurons (not shown), which in turn innervate motor neurons, which facilitate locomotory responses.

Chalasani, S.H., Chronis, N., Tsunozaki, M., Gray, J.M., Ramot, D., Goodman, M.B., and Bargmann, C.I. (2007). Dissecting a circuit for olfactory behaviour in Caenorhabditis elegans. Nature *450*, 63–70.

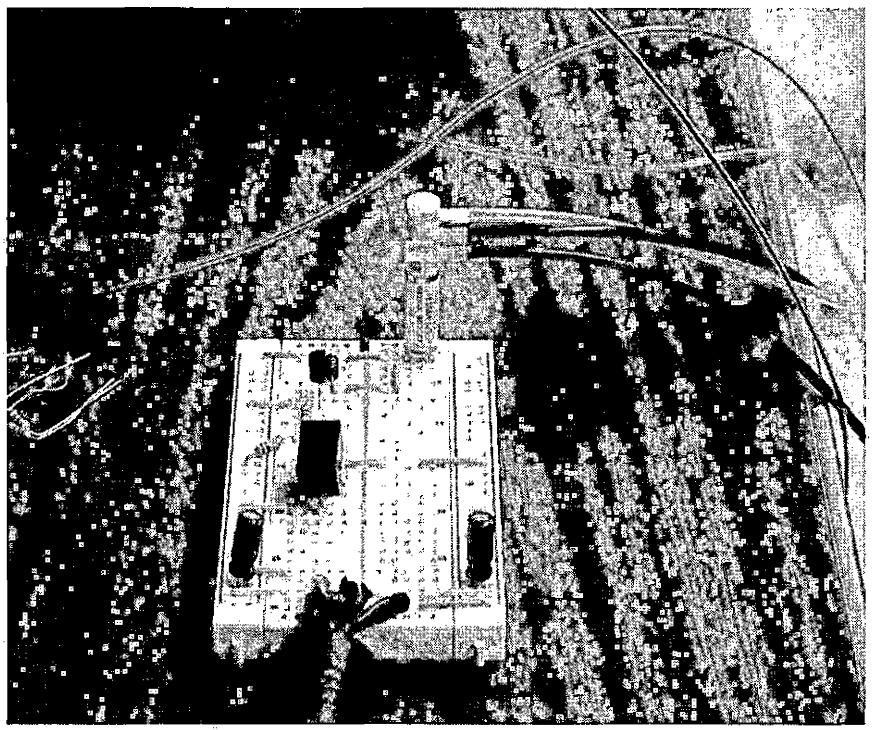

Figure 7. A Photo of the breadboard solenoid circuit initially used in microfluidics experiments. The valve control system utilized to control flow through the two dye channels. This system was used initially while performing preliminary experiments utilizing the microfluidics chip.

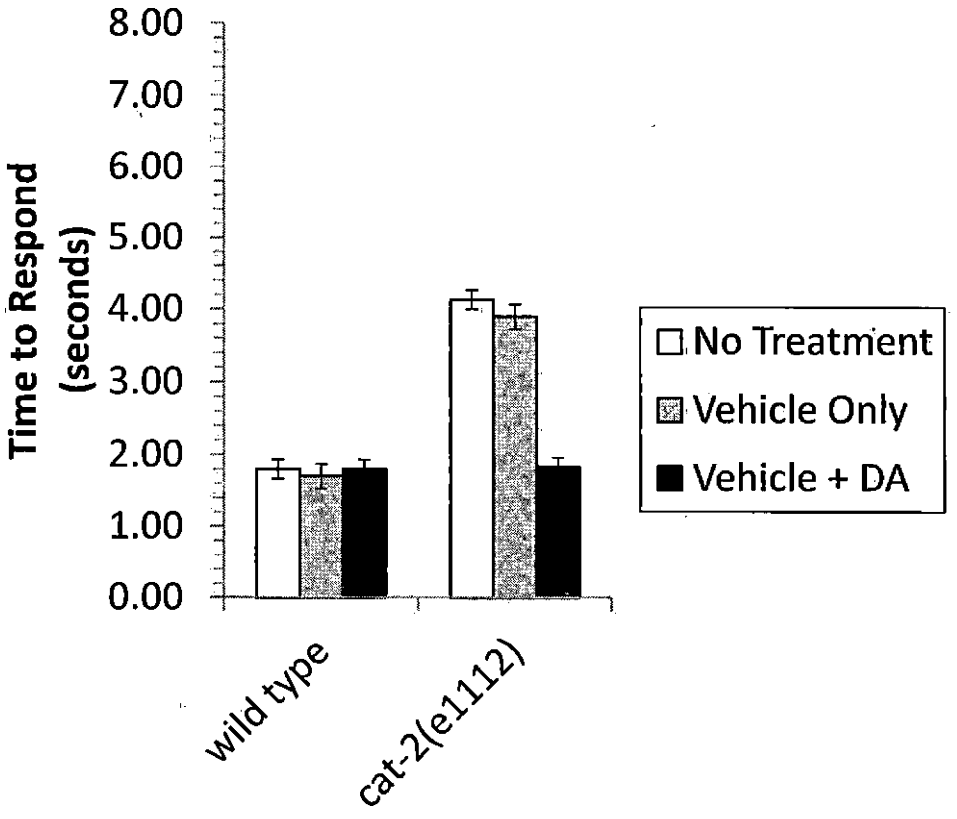

Figure 8. C. elegans response to 100% 1-octanol is modulated by dopamine. Animals were tested on standard NGM plates, NGM with vehicle (40 mL H_2O), or NGM with dopamine (DA; 40 mL 1M dopamine). Time to reversal after stimulus was scored and reported as mean +/- S.E.M. n=30. Data from M. Genovez and M. Baidya, unpublished.

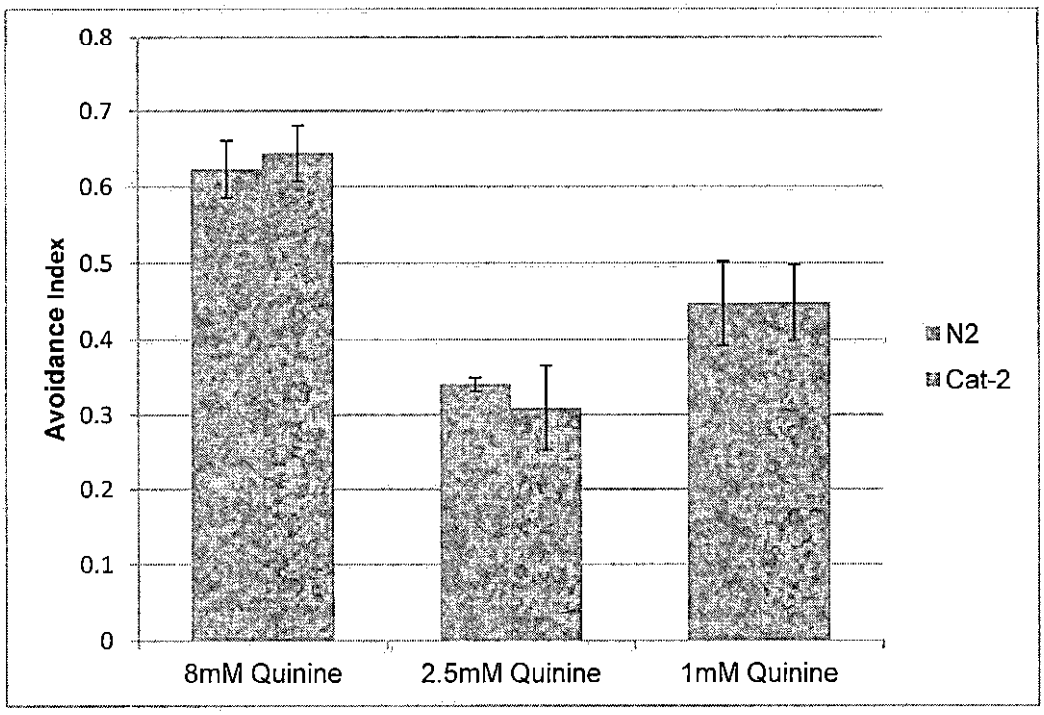

Figure 9. C. elegans response to quinine is not modulated by dopamine. Three different concentrations of quinine in M13 buffer were tested using the drop assay. Error bars indicate S.E.M. n=8-20. Avoidance index is defined as the proportion of worms responding in less than two seconds compared to total number of worms tested.

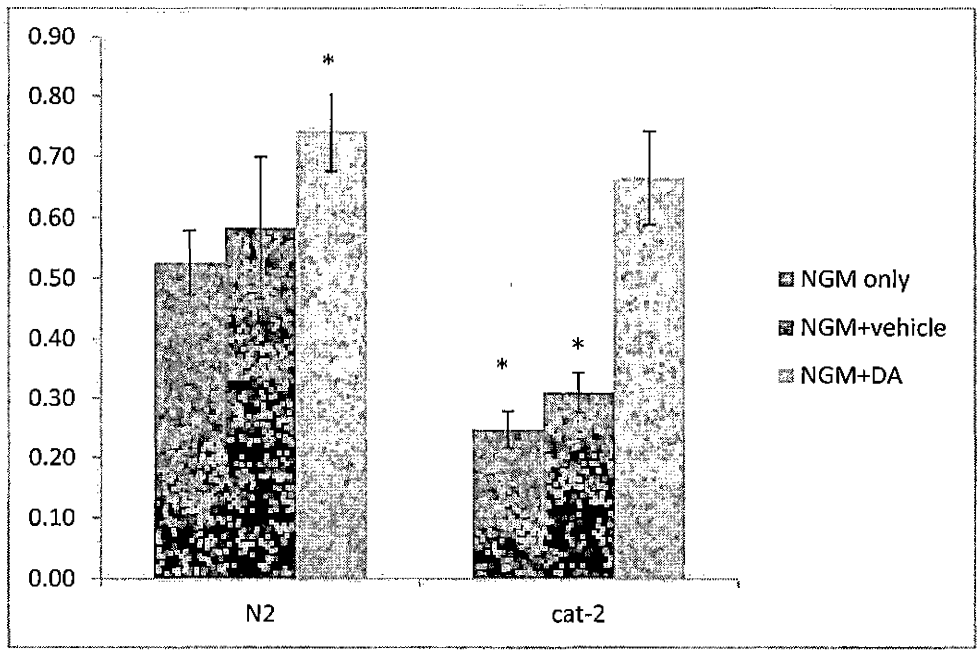

Figure 10. C. elegans response to SDS is modulated by dopamine. Animals were tested for response to 0.1% SDS in M13 buffer on standard NGM plates, NGM with vehicle (40 mL H_2O), or NGM with dopamine (DA; 40 mL 1M dopamine). Error bars indicate S.E.M. n=8~10. *p<0.05.

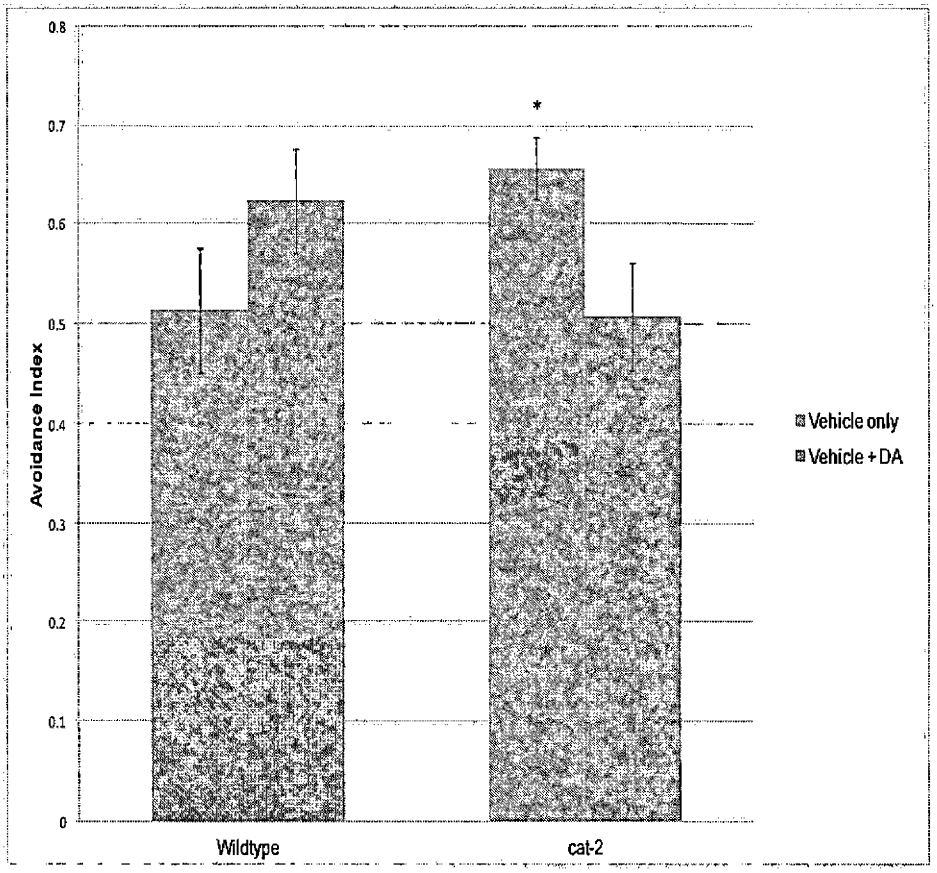

Figure 11. C. elegans response to glycerol is modulated by dopamine. Animals were tested for response to 1M glycerol in M13 buffer on standard NGM plates with vehicle (40 mL H_2O), or NGM with dopamine (DA; 40 mL 1M dopamine). Error bars indicate S.E.M. n=10. *p<0.06

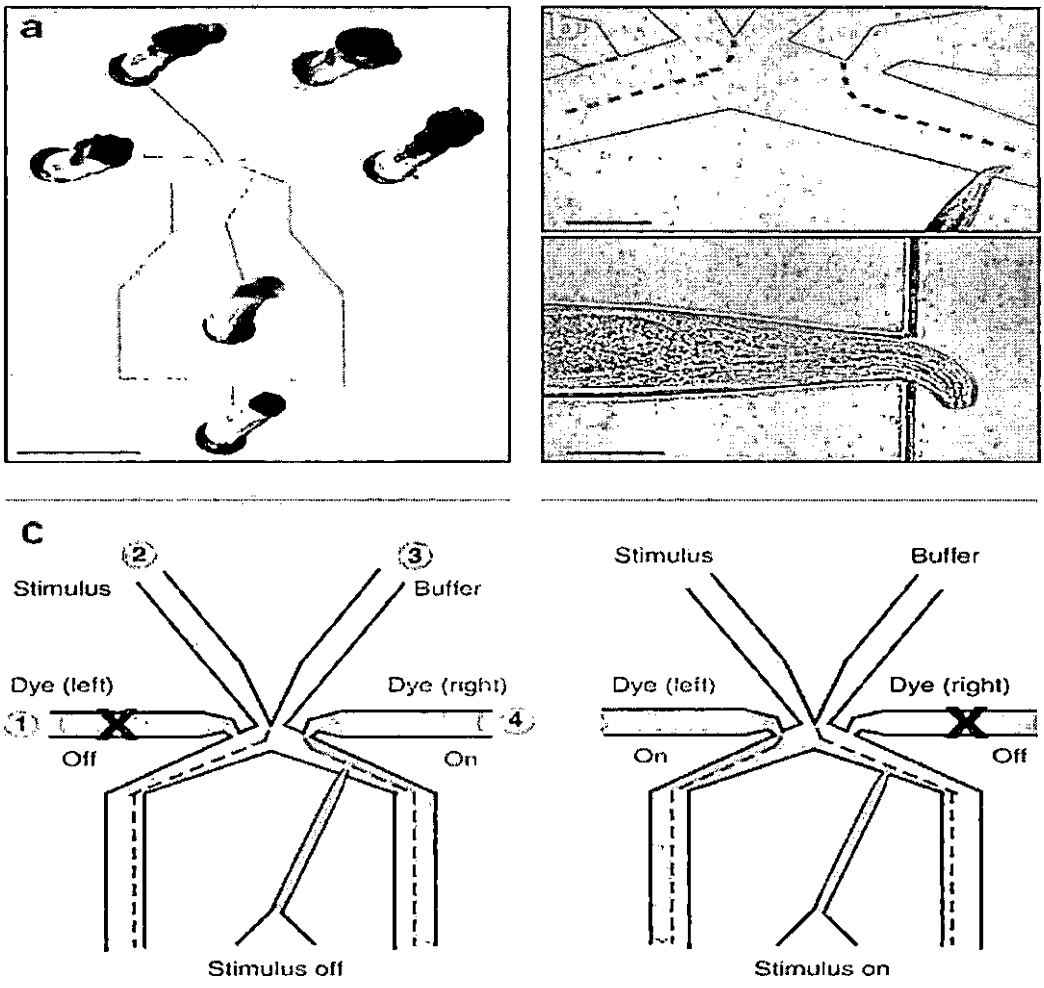

Figure 12. Schematic of the olfactory chip. (A) Micrograph of the olfactory chip with metal tubing inserted into each of the inlet ports as well as the vacuum port. (B) Close up of a worm inserted in the worm trap of the chip. (C) Diagram showing the mechanism of stimulus diversion using the dye channels. For details, see text.

Chronis, N., Zimmer, M., and Bargmann, C.I. (2007). Microfluidics for in vivo imaging of neuronal and behavioral activity in Caenorhabditis elegans. Nature Methods *4*, 727–731.

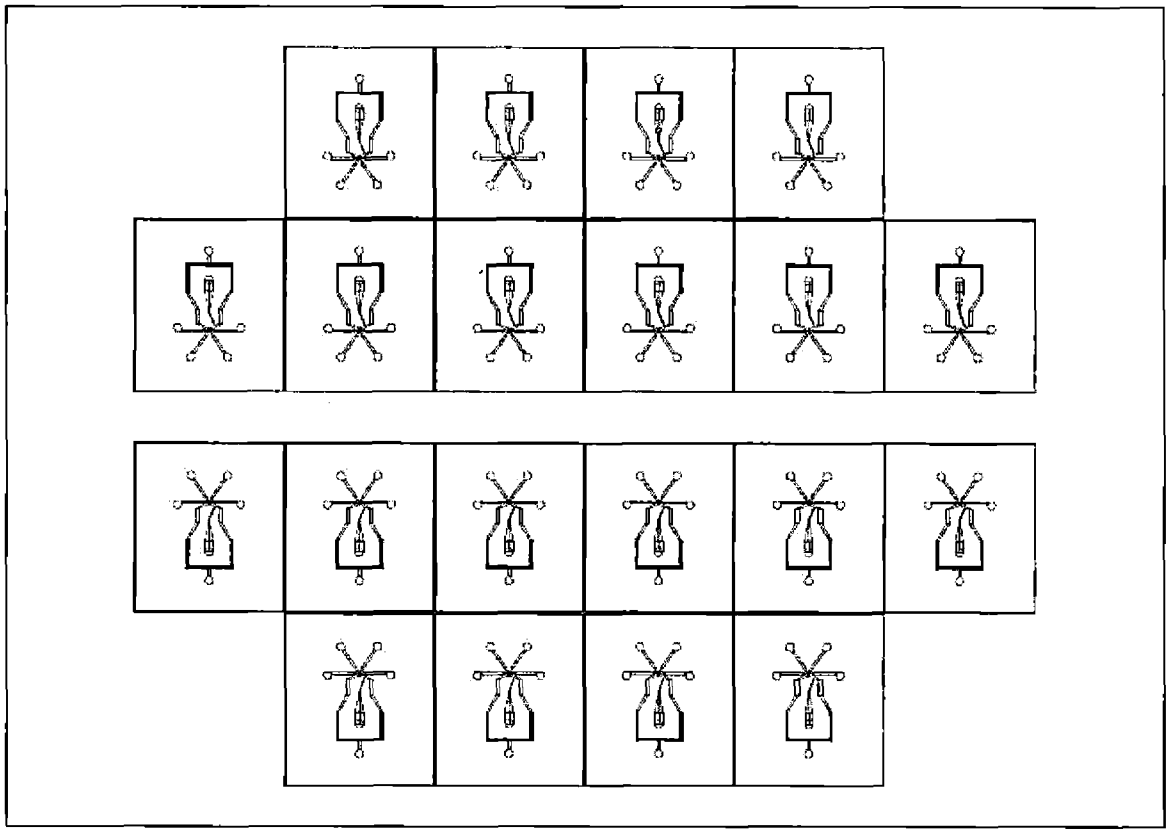

*Figure 13.*AutoCAD file containing the blueprint for the olfactory chip. Image of the AutoCAD file (a generous gift from S. Chalasani, Salk Institute) used to produce the olfactory chip mask. This design is suitable for casting within a 150 mm Petri dish, and produces 20 chips per casting. The dimensions of each chip are 10 mm x 10 mm.

Figure 14. Utilizing a coring tool to punch an inlet channel. An inlet port is being punched utilizing a .75 mm Harris Uni-Core tool. This tool not only punches the holes in the chip but cores them as well. In this way the inlet ports are produced.

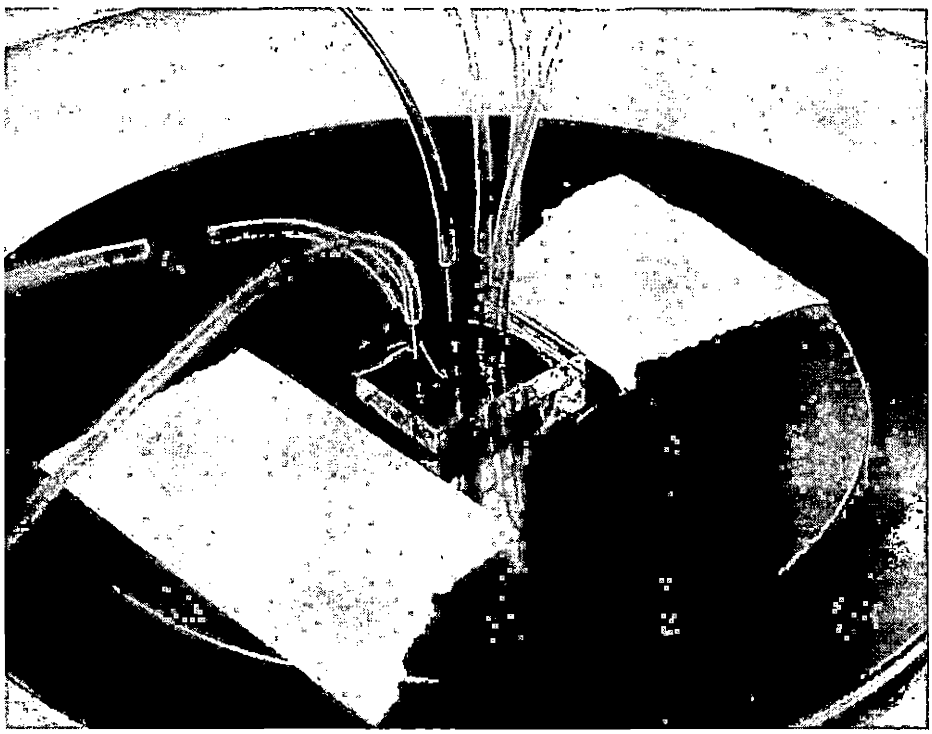

Figure 15. Olfactory chip interfaced with vacuum and fluid reservoirs. The chip is held on in place on the stage with labeling tape (green).

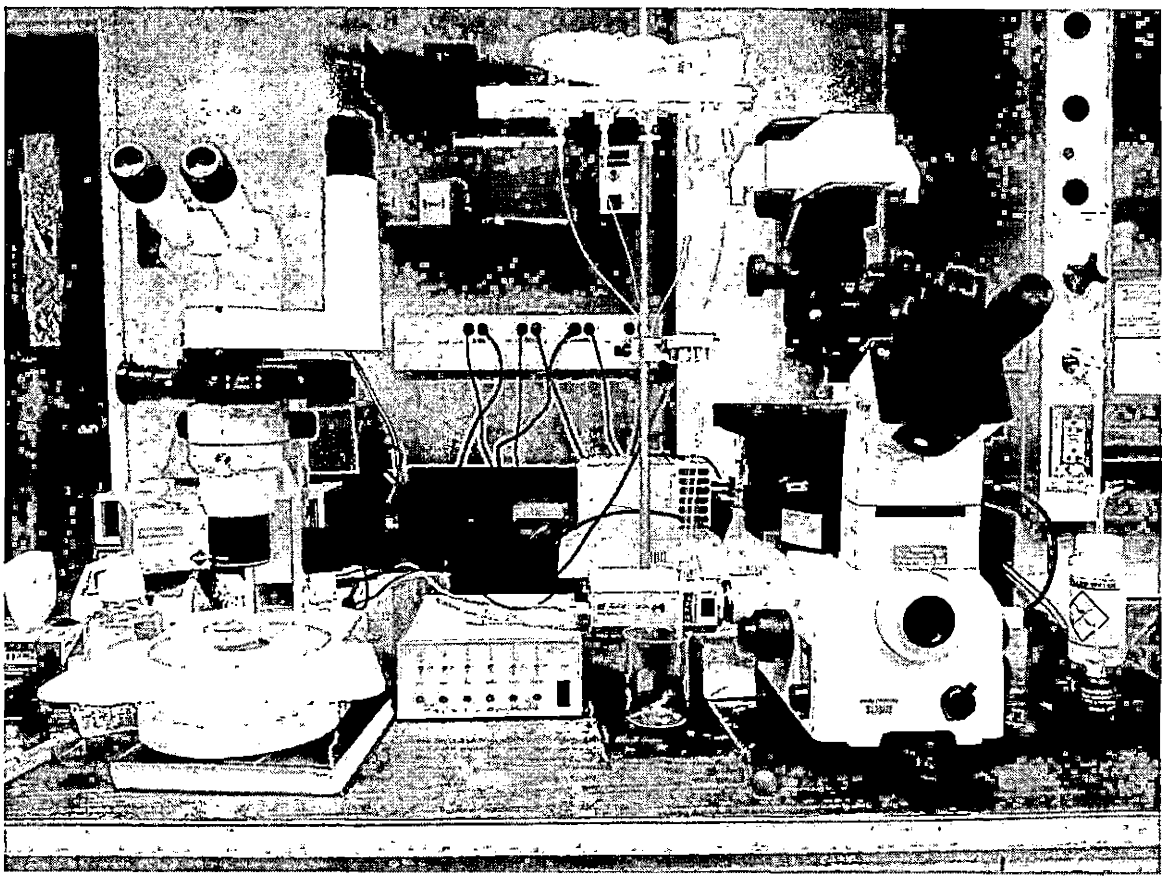

Figure 16. The Olfactory chip rig fully assembled. Shows both the inverted fluorescent microscope and stereomicroscope which are used in setting up the microfluidics rig. Includes the four fluid reservoirs, the solenoid valve and valve controller.

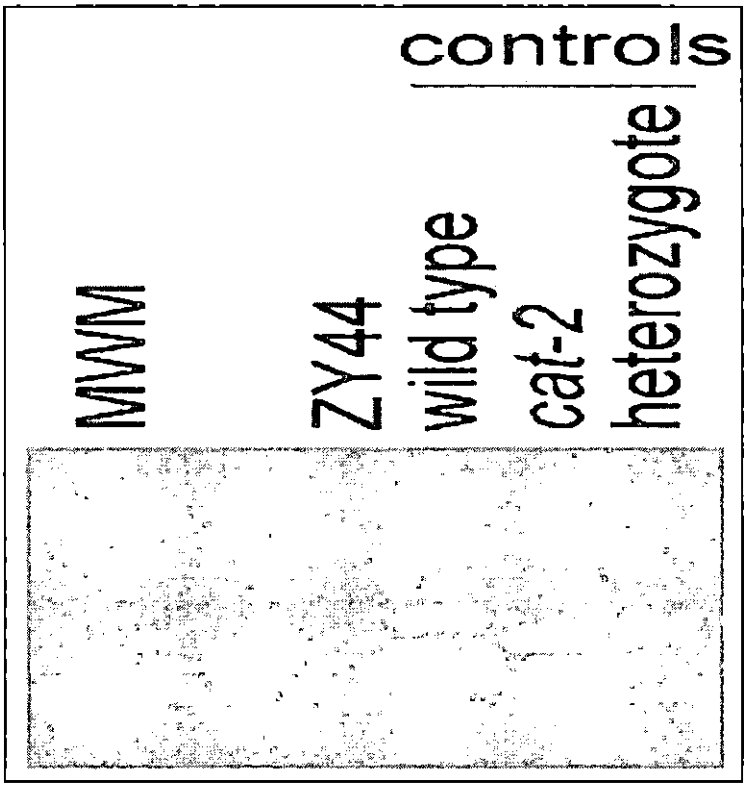

Figure 17. ZY44 is a cat-2 homozygote. The gel showing that the strain ZY44 is homozygous for the *cat-2* allele. The right most well contains a molecular weight ladder which contains DNA strands of known length; this can be used to approximate length of your DNA strand of interest. The third lane contains the newly produced *cat-2* strain, the fourth the homozygous wild type control, the fifth the homozygous *cat-2* control, and the sixth the heterozygous control.

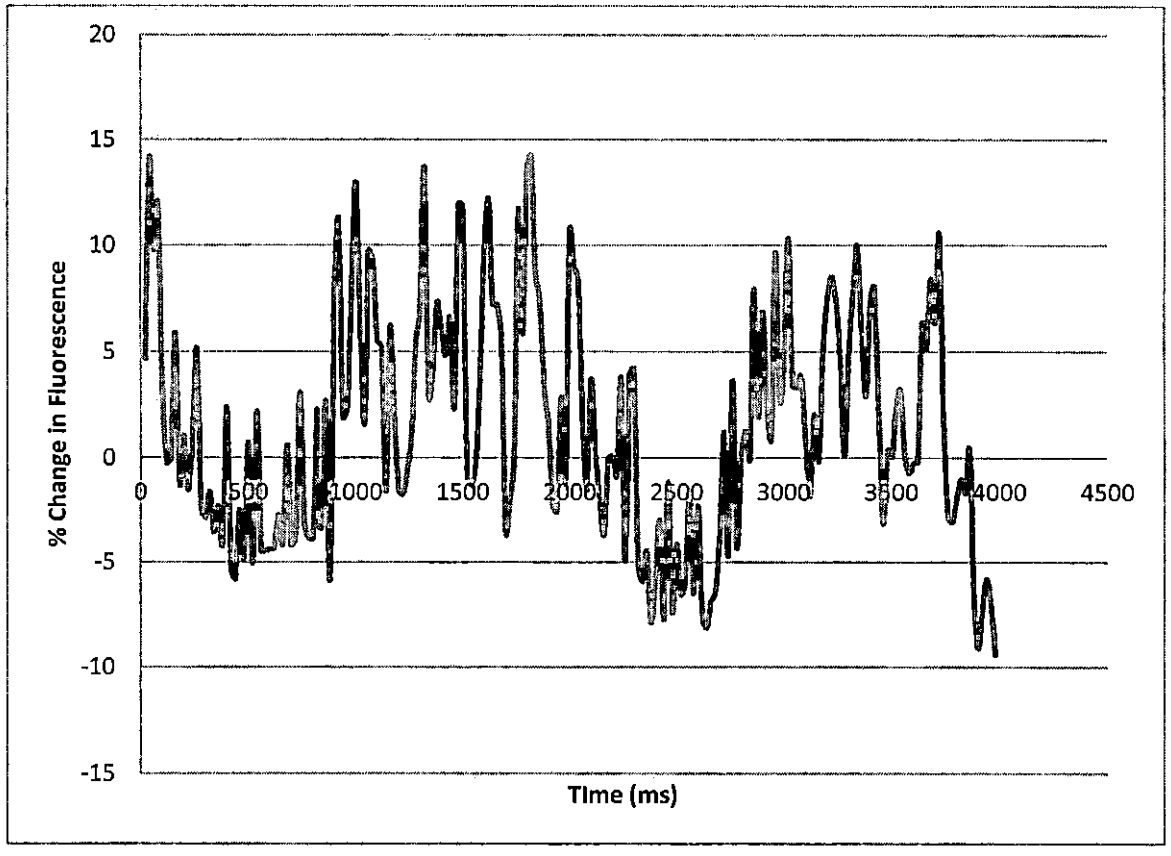

Figure 18. Ca^{2+} transients recorded from the *C. elegans* pharyngeal terminal bulb. Data are plotted as percent change in fluorescence over baseline as a function of time. A representative trace is shown. Pharyngeal pumping typically started after approx. 50 sec. of application of 25 mM serotonin (data not shown; see text for details).

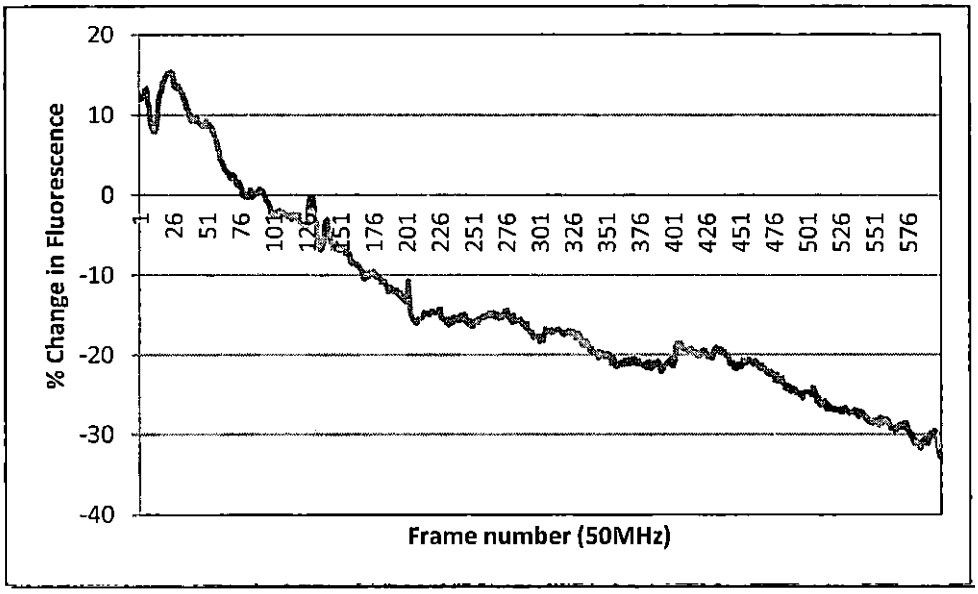

Figure 19. ASH exhibits no activity upon exposure to SDS. The above trace shows the average response of the seven wild type worms tested. The worms were exposed to the stimulus beginning at frame 200 and the stimulus was removed at frame 400. The ASH neuron showed no response to both exposure and removal of stimulus.

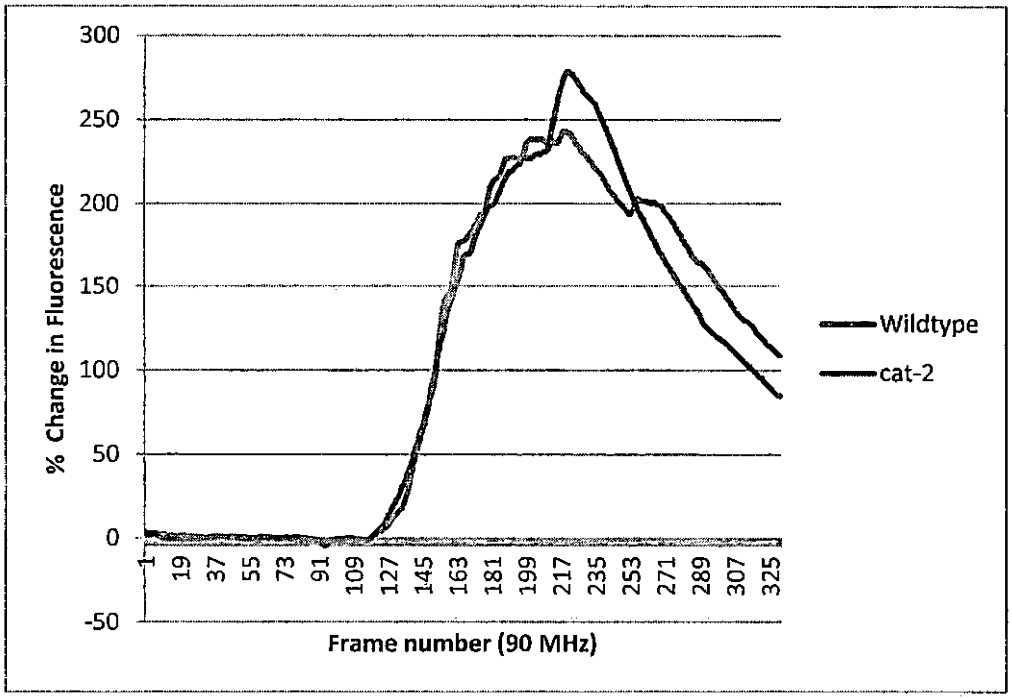

Figure 20. The response of ASH to glycerol is modulated by dopamine. The above graph shows calcium transients of both wild type and c*at-2* mutant worms in response to glycerol. The *cat-2* mutant worms showed an increased response to the removal of stimulus as compared to wild type worms. This difference was found to be statistically significant (see text for details on statistical analysis; n=7 for wild type, n=11 for *cat-2*).

WORK CITED

Alkema, M.J., Hunter-Ensor, M., Ringstad, N., Horvitz, H.R. (2005). Tyramine Functions Independently of Octopamine in the *Caenorhabditis elegans* Nervous System. *Neuron, 46*, 247–260.

Bargmann, C.I., and Averyt, L. (1995). Laser killing of cells in Caenorhabditis elegans. *Methods in Cell Biology, 48*, 225-250.

Bargmann, C.I., Hartwieg, E., and Horvitz, H.R. (1993). Odorant-selective genes and neurons mediate olfaction in C. elegans. *Cell,74*, 515–527.

Caron, M.G., Beaulieu, M., Raymond, V., Gagne, B., Drouin, J., and Lefkowitz R.J., Labrie, F., (1978). Receptors in the Anterior Pituitary Gland. *Journal of Biological Chemistry, 235(7),* 2244–2253.

Chalasani, S.H., Chronis, N., Tsunozaki, M., Gray, J.M., Ramot, D., Goodman, M.B., and Bargmann, C.I. (2007). Dissecting a circuit for olfactory behaviour in Caenorhabditis elegans. *Nature, 450(3)*, 63–70.

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Chao, M.Y., Komatsu, H., Fukuto, H.S., Dionne, H.M., and Hart, A.C. (2004). Feeding status and serotonin rapidly and reversibly modulate a Caenorhabditis elegans chemosensory circuit. *Proceedings of the National Academy of Sciences of the United States of America, 101 (43)*, 15512–15517.

Chase, D.L., Pepper, J.S., and Koelle, M.R. (2004). Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. *Nature Neuroscience, 7(10)*, 1096–1103.

Chia, N.-Y., Chan, Y.-S., Feng, B., Lu, X., Orlov, Y.L., Moreau, D., Kumar, P., Yang, L., Jiang, J., Lau, M.-S., et al. (2010). A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature, 468*(7321), 316–320.

Chiara, G. Di (1999). Drug addiction as dopamine-dependent associative learning disorder. *European Journal of Pharmocology, 375*(1), 13-30.

Chronis, N., Zimmer, M., and Bargmann, C.I. (2007). Microfluidics for in vivo imaging of neuronal and behavioral activity in Caenorhabditis elegans. *Nature Methods, 4*(9), 727–731.

Cools, R., Barker, R.A., Sahakian, B.J., and Robbins, T.W. (2001). Enhanced or impaired cognitive function in Parkinson's disease as a function of dopaminergic medication and task demands. *Cerebral Cortex,* 11(12), 1136-1143

Damier, P., Hirsch, E.C., Agid, Y., and Graybiel, A.M. (1999). The substantia nigra of the human brain II . Patterns of loss of dopamine-containing neurons in Parkinson ' s disease. *Brain,122,* 1437–1448.

De Bono, M., Tobin, D.M., Davis, M.W., Avery, L., and Bargmann, C.I. (2002). Social feeding in Caenorhabditis elegans is induced by neurons that detect aversive stimuli. *Nature, 419* , 899–903.

Duffy, D.C., McDonald, J.C., Schueller, O.J., and Whitesides, G.M. (1998). Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Analytical Chemistry*, *70*, 4974–4984.

Evans, A.H., Lawrence, A.D., Potts, J., MacGregor, L., Katzenschlager, R., Shaw, K., Zijlmans, J., and Lees, A.J. (2006a). Relationship between impulsive sensation seeking traits, smoking, alcohol, and caffeine intake, and Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *77(3)*, 317–321.

Evans, A.H., Pavese, N., Lawrence, A.D., Tai, Y.F., Appel, S., Doder, M., Brooks, D.J., and Lees, A.J. (2006b). Compulsive Drug Use Linked to Sensitized Ventral Striatal Dopamine Transmission. *Annals of Neurology*, *2*, 852–858.

Ezak, M.J., and Ferkey, D.M. (2010). The C. elegans D2-like dopamine receptor DOP-3 decreases behavioral sensitivity to the olfactory stimulus 1-octanol. *PloS One, 5(3)*, e9487.

Ferkey, D.M., Hyde, R., Haspel, G., Dionne, H.M., Hess, H. a, Suzuki, H., Schafer, W.R., Koelle, M.R., and Hart, A.C. (2007). C. elegans G protein regulator RGS-3 controls sensitivity to sensory stimuli. *Neuron, 53(1)*, 39–52.

Guo, Z. V, Hart, A.C., and Ramanathan, S. (2009). Optical interrogation of neural circuits in Caenorhabditis elegans. *Nature methods*, *6*(12), 891-896.

Harris, G.P., Hapiak, V.M., Wragg, R.T., Miller, S.B., Hughes, L.J., Hobson, R.J., Steven, R., Bamber, B., and Komuniecki, R.W. (2009). Three distinct amine receptors operating at different levels within the locomotory circuit are each essential for the serotonergic modulation of chemosensation in

Caenorhabditis elegans. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 29(5)*, 1446–1456.

Hart, A.C., Kass, J., Shapiro, J.E., and Kaplan, J.M. (1999). Distinct signaling pathways mediate touch and osmosensory responses in a polymodal sensory neuron. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 19(6)*, 1952–1958.

Haubert, K., Drier, T., and Beebe, D. (2006). PDMS bonding by means of a portable, low-cost corona system. *Lab on a Chip, 6(12)*, 1548–1549.

Hemmings, H.C., Greengard, P., and Tung, H. Y. Lim Cohen, P. (1984). DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature, 310(5977)*, 503–505.

Hilliard, M. a, Bargmann, C.I., and Bazzicalupo, P. (2002). C. elegans responds to chemical repellents by integrating sensory inputs from the head and the tail. *Current Biology, 12(9)*, 730–734.

Hilliard, M. a, Bergamasco, C., Arbucci, S., Plasterk, R.H. a, and Bazzicalupo, P. (2004a). Worms taste bitter: ASH neurons, QUI-1, GPA-3 and ODR-3 mediate quinine avoidance in Caenorhabditis elegans. *The EMBO Journal ,23(5)*, 1101–1111.

Hilliard, M. a, Apicella, A.J., Kerr, R., Suzuki, H., Bazzicalupo, P., and Schafer, W.R. (2004b). In vivo imaging of C. elegans ASH neurons: cellular response and adaptation to chemical repellents. *The EMBO Journal, 24(1)*, 63–72.

Hills, T., Brockie, P.J., and Maricq, A.V. (2004). Dopamine and glutamate control area-restricted search behavior in Caenorhabditis elegans. *The Journal of Neuroscience, 24(5)*, 1217.

Huang, B., Wu, H., Bhaya, D., Grossman, A., Granier, S., Kobilka, B.K., and Zare, R.N. (2007). Counting Low-Copy Number Proteins in a Single Cell. *Science, 315(5808)* , 81–84.

Kahn-Kirby, A.H., Dantzker, J.L.M., Apicella, A.J., Schafer, W.R., Browse, J., Bargmann, C.I., and Watts, J.L. (2004). Specific polyunsaturated fatty acids drive TRPV-dependent sensory signaling in vivo. *Cell, 119(6)*, 889–900.

Kaplan, J.M., and Horvitz, H.R. (1993). A dual mechanosensory and chemosensory neuron in Caenorhabditis elegans. *Proceedings of the*

National Academy of Sciences of the United States of America, 90(6), 2227–2231.

Kebabian, J.W., and Calne, D.B. (1979). Multiple receptors for dopamine. *Nature, 27(5692)7*, 93–96.

Kindt, K.S., Quast, K.B., Giles, A.C., De, S., Hendrey, D., Nicastro, I., Rankin, C.H., and Schafer, W.R. (2007). Dopamine mediates context-dependent modulation of sensory plasticity in C. elegans. *Neuron, 55*(4), 662–676.

Lagally, E.T., Medintz, I., and Mathies, R.A. (2001). Single-Molecule DNA Amplification and Analysis in an Integrated Microfluidic Device. *Analytical Chemistry,73(3)*, 565–570.

Lee, R.Y., Sawin, E.R., Chalfie, M., Horvitz, H.R., and Avery, L. (1999). EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in caenorhabditis elegans. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 19(1)*, 159–167.

Leyton, M., Boileau, I., Benkelfat, C., Diksic, M., Baker, G., and Dagher, A. (2002). Amphetamine-induced increases in extracellular dopamine, drug wanting, and novelty seeking: a PET/[11C]raclopride study in healthy men. *Neuropsychopharmacology* : Official Publication of the American College of Neuropsychopharmacology, *27(6)*, 1027–1035.

Lotharius, J., and Brundin, P. (2002). Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nature Reviews. Neuroscience, 3(12)*, 932–942.

Mamikonyan, E., Siderowf, A.D., Duda, J.E., Potenza, M.N., Horn, S., Stern, M.B., and Weintraub, D. (2008). Long-term follow-up of impulse control disorders in Parkinson's disease. *Movement Disorders Official Journal of the Movement Disorder Society, 23(1)*, 75–80.

Medintz, I. (2001). High-Performance Multiplex SNP Analysis of Three Hemochromatosis-Related Mutations With Capillary Array Electrophoresis Microplates. *Genome Research, 11(3)*, 413–421.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *The EMBO Journal,10(1)*, 3959–3970.

Goodman, M.,B., Hall,D.,H., Avery,L., Lockery, S.,R., (1998). Active Currents Regulate Sensitivity and Dynamic Range in C. elegans Neurons. *Neuron, 20(4)*, 763–772.

Kareiva,P., Odell, G., (2013). Swarms of predators exhibit "preytaxis" if individual predators use area restricted search. *The American Naturalist, 130(2)*, 233–270.

Lints,R., Emmons,S.,W., (1998). cat-2 encodes a putative tyrosine hydroxylase that is essential for dopamine biosynthesis. *Worm Breeder's Gazette, 15.*

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.,M., Adams, J.,A., Ikura, M., Tsien,R..Y., (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature, 388(6645)*, 882–887.

Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nature Biotechnology, 19(2)*, 137–141.

Niacaris, T. (2003). Serotonin regulates repolarization of the C. elegans pharyngeal muscle. *Journal of Experimental Biology, 206(2)*, 223–231.

Robbins, T.W., and Everitt, B.J. (1999). Drug addiction: bad habits add up. *Nature, 398(6728)*, 567–570.

Sanyal, S., Wintle, R.F., Kindt, K.S., Nuttley, W.M., Arvan, R., Fitzmaurice, P., Bigras, E., Merz, D.C., Hébert, T.E., Van der Kooy, D., et al. (2004). Dopamine modulates the plasticity of mechanosensory responses in Caenorhabditis elegans. *The EMBO Journal, 23(2)*, 473–482.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron, 26(3)*, 619–631.

Schultz, W. (2006). Behavioral theories and the neurophysiology of reward. *Review Literature And Arts Of The Americas,57,* 87–115.

Sugiura, M., Fuke, S., Suo, S., Sasagawa, N., Tol, Hubert, H.,M., and Ishiura, S. (2005). Characterization of a novel D2-like dopamine receptor with a truncated splice variant and a D1-like dopamine receptor unique to invertebrates from Caenorhabditis elegans. *Journal of Neurochemistry,* 94(4), 1146–1157.

Sulston, J. E., & Brenner, S. (1974). The DNA of Caenorhabditis elegans.*Genetics*, *77*(1), 95-104.

Sulston, J., Dew, M., and Brenner, S. (1975). Dopaminergic neurons in the nematode Caenorhabditis elegans. *The Journal of Comparative Neurology, 163(2)*, 215–226.

Sulston, J., & Hodgkin, J. (1988). Methods. *Cold Spring Harbor Monograph Archive, 17*(0). Retrieved May 29, 2013, from https://cshmonographs.org/index.php/monographs/article/view/5032

Suo, S., Sasagawa, N., and Ishiura, S. (2002). Identification of a dopamine receptor from Caenorhabditis elegans. *Neuroscience Letters, 319(1)*, 13–16.

Todes, C.J., and Lees, A.J. (1985). The pre-morbid personality of patients with Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry, 48(2)*, 97–100.

Troemel, E.R. (1999). Chemosensory signaling in C. elegans. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology, 21(12)*, 1011–1020.

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H. A., and Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in C. elegans. *Cell, 83(2)*, 207–218.

Troemel, E.R., Kimmel, B.E., and Bargmann, C.I. (1997). Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in C. elegans. *Cell, 91(2)*, 161–169.

Truong, K., Sawano, A., Mizuno, H., Hama, H., Tong, K.I., Mal, T.K., Miyawaki, A, and Ikura, M. (2001). FRET-based in vivo Ca2+ imaging by a new calmodulin-GFP fusion molecule. *Nature Structural Biology, 8(12)*, 1069–1073.

Urbanski, J.P., Johnson, M.T., Craig, D.D., Potter, D.L., Gardner, D.K., and Thorsen, T. (2008). Noninvasive Metabolic Profiling Using Microfluidics for Analysis of Single Preimplantation Embryos. *Analytical Chemistry, 80(17)*, 6500–6507.

Walker, R.G. (2000). A Drosophila Mechanosensory Transduction Channel. *Science, 287(5461)*, 2229–2234.

Wang, Y., Wang, J., Du, W., Feng, X.J., and Liu, B.F. (2011). Identification of the neuronal effects of ethanol on C. elegans by in vivo fluorescence imaging on a microfluidic chip. *Analytical and Bioanalytical Chemistry, 399(10)*, 3475–3481.

Wei Li,X., Feng,Z., Strenberg, P., X.Z.S.X. (2006). A C.elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. *Nature, 440(7084)*, 684–687.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The Structure of the Nervous System of the Nematode Caenorhabditis elegans. *Philosophical Transactions of the Royal Society of London. Biological Sciences, 314* , 1–340.

Wise, R. (2004). Dopamine, learning and motivation. *Nature Reviews. Neuroscience, 5(6)*, 483–494.

Wragg, R.T., Hapiak, V., Miller, S.B., Harris, G.P., Gray, J., Komuniecki, P.R., and Komuniecki, R.W. (2007). Tyramine and octopamine independently inhibit serotonin-stimulated aversive behaviors in Caenorhabditis elegans through two novel amine receptors. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 27(49)*, 13402–13412.

Yang, X.L. (2004). Characterization of receptors for glutamate and GABA in retinal neurons. *Progress in Neurobiology, 73(2)*, 127–150.

Zetterström, T., Sharp, T., Marsden, C.A., and Ungerstedt, U. (1983). In Vivo Measurement of Dopamine and Its Metabolites by Intracerebral Dialysis: Changes After d-Amphetamine. *Journal of Neurochemistry, 41(6)*, 1769–1773.

Zhang, X., and Cote, R.H. (2005). cGMP signalling in vertebrate retinal photoreceptor cells.*Front Biosci, 10,* 1191–1204.