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Glutamate Transporters in Caenorhabditis Elegans: the Implications of Transporter Deletions on Behavior, Learning, Memory, and Addiction

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Glutamate Transporters in *Caenorhabditis Elegans*: the Implications of Transporter Deletions on Behavior, Learning, Memory, and Addiction

Moriah Harling
Submitted in Partial Fulfillment of the Prerequisite for Honors in Neuroscience under the advisement of Deborah Bauer

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1 Acknowledgements

This thesis provides a detailed account of the completed work pertaining to the learning and memory project of the Bauer Lab. All protocols, as well as the direction of the project, were designed and developed by myself in collaboration with my research advisor, Deborah Bauer. Multiple members of the Bauer Lab contributed to this project through conducting experimental replicates for the various sections.

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2 Abstract

Glutamate is a critical neurotransmitter involved in excitatory synaptic transmission, cognition, memory, and learning. Although much research has been conducted to examine glutamatergic signaling pathways, the functional role of glutamate transporters (GLTs) in behavior, learning, and memory remains largely unexplored in Caenorhabditis elegans. We tested wildtype C. elegans and C. elegans with GLT deletions on a battery of behavioral tests including analysis of spontaneous locomotion, response to a mechanosensory stimulus, response to an aversive chemical, and chemotaxis in response to a chemoattractant and a chemorepellent to examine how different transporters affect basic behaviors. Thus far, we have examined C. elegans with deletions of GLT-3 located in canal cells, GLT-1 located in muscle cells, GLT-4 located on presynaptic neurons, and GLT-5 located within the pharyngeal region. We then conducted associative and non-associative learning paradigms. Compared to wildtypes, GLT-3; GLT-1 knockouts were deficient in all basic behavioral tasks besides chemotaxis in response to a chemorepellent, GLT-4 knockouts were deficient in all basic behavioral tasks besides response to the smell of an aversive chemical, and GLT-5 knockouts were deficient in response to the smell of an aversive chemical and chemotaxis in response to a chemoattractant, however they exhibited a hyperactive chemotactic response to a chemorepellent. All mutants displayed associative learning, however GLT-4 and GLT-5 mutants had less of an extreme response to the aversive chemical prior to conditioning and expressed less association to that same chemical in the post-learning period. Mutants with GLT-3; GLT-1 or GLT-5 deletions do not display normal habituation, a type of non-associative learning, while GLT-4 mutants were not analyzed in this paradigm due to an abnormal initial response. We next examined exposure to the addictive substances nicotine and ethanol as a type of memory. We evaluated the ability for different mutant strains to express drug-seeking behavior and initial preference for these drugs. We then tested whether the mutants would associate the drugs with an aversive chemical as we demonstrated in wildtypes. Compared to wildtypes, all mutant strains expressed decreased chemoattraction toward ethanol but unaltered chemoattraction toward nicotine. Furthermore, all mutants exhibited associative learning after ethanol or nicotine conditioning but expressed less association in the post-learning period than the wildtype strain. Our data suggest that basic behaviors may rely on different glutamate transporters than learning and memory do. These differences may be attributable to differences in transporter localization.
# Contents

1 Acknowledgements 1

2 Abstract 2

3 Introduction 5
   3.1 Introduction to Glutamate .................................. 5
   3.2 The Glutamate Synapse .................................... 6
   3.3 Glutamate’s Role in Behavior, Learning, and Memory ...... 7
   3.4 *C. elegans* as a Model Organism ............................ 9
   3.5 GLT in *C. elegans* ...................................... 10
   3.6 *C. elegans* and GLT Research ............................... 11
   3.7 Research Questions ........................................ 11

4 Methods 13
   4.1 Animals ................................................... 13
   4.2 Basic Behaviors .......................................... 13
      4.2.1 Nose Touch ........................................... 13
      4.2.2 Reversal Time ....................................... 13
      4.2.3 Smell-on-a-Stick ................................... 13
      4.2.4 Chemotaxis (Chemoattraction and Chemorepulsion) ... 13
   4.3 Associative Learning ....................................... 14
      4.3.1 Chemotaxis in Response to Butanone .................. 14
      4.3.2 Association Control Paradigm ......................... 15
      4.3.3 Association Paradigm ................................ 15
   4.4 Non-Associative Learning ................................. 16
      4.4.1 Short-Term Tapping Habituation ....................... 16
      4.4.2 Long-Term Tapping Habituation (To be completed) ... 17
   4.5 Addiction Paradigms ...................................... 17
      4.5.1 Chemotaxis in Response to Ethanol and Nicotine ...... 17
      4.5.2 Ethanol Association Paradigm ........................ 17
      4.5.3 Nicotine Association Paradigm ....................... 18
      4.5.4 Habituation to Nicotine (To be completed) .......... 18
   4.6 Statistical Analysis ...................................... 19
      4.6.1 Analysis of Basic Behaviors, Associative Learning, and Addiction Paradigms ........................................ 19
      4.6.2 Analysis of Non-Associative Learning Paradigm ....... 19

5 Results 20
   5.1 Basic Behaviors .......................................... 20
      5.1.1 Nose Touch ........................................... 20
      5.1.2 Reversal Time ....................................... 21
      5.1.3 Smell-on-a-Stick ................................... 21
      5.1.4 Chemotaxis (Chemoattraction and Chemorepulsion) ... 22
   5.2 Associative Learning ..................................... 24
5.2.1 Association Control Paradigm ........................................ 24
5.2.2 Association Paradigm .................................................... 25
5.3 Non-Associative Learning .................................................. 27
  5.3.1 Short-Term Tapping Habituation ................................... 27
5.4 Addiction Paradigms ....................................................... 29
  5.4.1 Ethanol Chemotaxis .................................................... 29
  5.4.2 Ethanol Associative Learning ........................................ 29
  5.4.3 Nicotine Chemotaxis ................................................... 30
  5.4.4 Nicotine Associative Learning ....................................... 31

6 Discussion ........................................................................... 33
  6.1 Part 1: Implications of GLT Deletions on Basic Behaviors ....... 33
  6.2 Part 2: Implications of GLT Deletions on Associative Learning ... 34
  6.3 Part 3: Implications of GLT Deletions on Non-Associative Learning ... 35
  6.4 Part 4: Implications of GLT Deletions on Addiction .................. 36
  6.5 Conclusions ..................................................................... 37
  6.6 Future Directions ............................................................ 38

7 Literature Cited ...................................................................... 40

8 Appendix ................................................................................ 45
  8.1 Data Visualization and Analysis for Basic Behaviors, Associative Learning, and Addiction (R Code) .... 45
  8.2 Data Visualization and Analysis for Non-Associative Learning (R Code) ............................................... 61
  8.3 Arduino Code to Control Mechanical Tapper ....................... 70
    8.3.1 Training Phase .......................................................... 70
    8.3.2 Testing Phase ........................................................... 71
3 Introduction

3.1 Introduction to Glutamate

Glutamate ($C_5H_8NO_4^-$) is a major neurotransmitter involved in excitatory synaptic transmission and has been shown to be involved in many different processes including cognition, memory, and learning. It functions throughout the nervous system and is also found in high concentration throughout the body because it is an amino acid. Glutamate is the most abundant neurotransmitter in vertebrates and the most prevalent amino acid in humans (Schousboe, 1981). Glutamate is considered a non-essential amino acid as it does not need to be synthesized in the body and can be obtained through a traditional diet (Krebs, 1935). However, there are two main pathways by which glutamate can be synthesized in neurons. First, glutamine can be converted into glutamate though the enzyme glutaminase (Erecinska and Silver, 1990; Walls et al., 2015). Second, alpha-ketoglutarate, which is an intermediate product of the citric acid cycle, can be converted to glutamate through the enzyme glutamate dehydrogenase (Erecinska and Silver, 1990). Furthermore, glutamate is also the precursor to the major inhibitory neurotransmitter, gamma-Aminobutyric acid (GABA), which is synthesized from glutamate via the enzyme glutamic acid decarboxylase (Erecinska and Silver, 1990; Walls et al., 2015). Although glutamate is considered the major excitatory neurotransmitter across vertebrate species, it was not acknowledged for many years due to its high abundance throughout the body in conjunction with the widespread belief that neurotransmitters were only that and were not found or have functions in in other parts of the body besides the nervous system (Fonnum, 1984).
3.2 The Glutamate Synapse

The mammalian glutamate synapse includes presynaptic and postsynaptic neurons but is also surrounded by a type of glial cell called astrocytes (Ventura and Harris, 1999). Glutamate is released from the presynaptic cell and binds to receptors that are located primarily on the postsynaptic membrane, although some metabotropic receptors are located on the presynaptic membrane (Sheng and Kim, 2011; MacGillavry et al., 2011). There are three major classes of glutamate receptors (GLR), which include α-aminos-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-Methyl-D-aspartic acid (NMDA), and metabotropic receptors. There is also a fourth GLR subtype, called kainate receptors, which are much less common. The amount of glutamate within synapses must be precisely controlled to prevent excitotoxicity, or the overactivation of
receptors by excitatory neurotransmitters such as glutamate, from occurring and leading to neuronal damage or death (Olney, 1969; Rothman, 1964). Excitotoxicity has been implicated in many neurodegenerative diseases affecting memory and cognition in humans (Lau and Tymianski, 2010). Glutamate is removed from the synaptic cleft via highly specialized proteins called glutamate transporters (GLT). This removal of glutamate from the synapse is a vital component to the health and efficacy of the glutamatergic system. The presence of GLT also ensures that the concentration of glutamate within the synapse remains precisely regulated to allow the signal being transported through the neuronal network to remain intact (Kanai and Hediger, 2003). In mammals the majority of glutamate is cleared from the synapse by GLT that are located on the surrounding astrocytes, where glutamate is often metabolized to glutamine (or in some cases α-ketoglutarate) and then transported to the presynaptic neurons to be transformed back into glutamate (Bergles and Jahr, 1997; Erecinska and Silver, 1990). A smaller, but still significant, portion of glutamate is taken up by GLT that are embedded in the postsynaptic membrane (Sheng and Kim, 2011).

3.3 Glutamate’s Role in Behavior, Learning, and Memory

Glutamate receptors modulate synaptic plasticity and appear to be an important mechanism for the formation of memory and learning as well as different behavioral activities. NMDA GLRs have been shown to be involved in the encoding of memories (Lee and Kim, 1998; Ekstrom et al., 2001). It is known that AMPA GLR deletion blocks neuronal communication, which indirectly affects the ability to learn and remember, but the exact mechanism by which AMPAR receptors are involved in memory remains unknown (Bassani et al., 2013). Perhaps the most important and direct way that glutamate is involved in memory is through the process of long-term potentiation (LTP). LTP is the strengthening of synapses that occurs after repeated high-frequency chemical stimulation of the synapse and has been implicated in memory formation. Multiple types of LTP exist but are often characterized as being either NMDA receptor-dependent or NMDA receptor-independent (Bliss and Collingridge, 1993; Vollianskis et al., 2015). NMDA receptor-dependent LTP is input specific, which prevents the induction of LTP at one synapse from spreading to other nearby synapses, and is persistent over a period of minutes to weeks (Vollianskis et al., 2015). In NMDA receptor-dependent LTP, concurrent activation of the presynaptic and postsynaptic glutamate neurons results in the activation of NMDA receptors (Bliss and Collingridge, 1993; Vollianskis et al., 2015). This activation then results in the redistribution of AMPA receptors across the postsynaptic membrane and modifies the strength of the synapse (Henley and Wilkinson, 2013; Vollianskis et al., 2015). Aberrant LTP mechanisms has been implicated in the loss of proper synaptic plasticity and the formation of neurodegenerative diseases such as Alzheimer’s disease and dementia (Henley and Wilkinson, 2013).

Besides its specific role in LTP, glutamate has been shown to mediate the processes of associative and nonassociative learning and memory (Riedel
et al., 2003). Associative learning is the ability for an organism to learn and remember a relationship between two distinct and unrelated objects (Hawkins and Byrne, 2015). Nonassociative learning is the persistent change in response to a stimulus after repeated exposure to said stimulus (Cerbone and Sadile, 1994). Glutamate has specifically been implicated in a type of nonassociative learning referred to as habituation (Cerbone and Sadile, 1994; Riedel et al., 2003). Habituation is the decrease of a response to a stimulus that under normal conditions elicits a noticeable and consistent behavioral or biological response (Cerbone and Sadile, 1994). Importantly, habituation is not due to the organism’s inability to respond but rather the learned behavior that a response is no longer needed or biologically functional.

Furthermore, glutamate has also been shown to modulate addiction through its role in the mesolimbic reward pathway. The mesolimbic reward pathway is a dopaminergic pathway that connects the ventral tegmental area (VTA) to the nucleus accumbens via the medial forebrain bundle and regulates the physiological and behavioral responses to rewarding stimuli (Ross et al., 2009b; Ross et al., 2009a; Trainor, 2011; Baracz et al., 2012). The pathway causes social, chemical, and biological rewards to initiate a motivational response and is involved in the perception of pleasure and gratification (Ross et al., 2009b; Ross et al., 2009a; Trainor, 2011). The function and cellular mechanisms of the pathway are conserved across most vertebrate species and remain vital in regulating day-to-day interactions with external stimuli (Trainor, 2011). However, the mesolimbic reward pathway is mostly acknowledged solely for its role in addictive disorders (Trainor, 2011). Addiction, which relies on both external stimuli as well as cellular and molecular signals, is mediated by the mesolimbic reward pathway (McGregor and Bowen, 2012; Lee et al., 2016; Lee and Weerts, 2016). The pathway associates the rewarding stimuli with a positive valence through increasing dopamine levels and reinforcement of the addictive behavior that results in repeated consumption or application of the stimulus (Trainor, 2011; McGregor and Bowen, 2012). Although the mesolimbic reward pathway is mostly dopamine based, glutamate plays an important role in modulating the function of dopaminergic neurons (Jo et al., 1998; Tzschentke and Schmidt, 2003; Qi et al., 2009). Glutamatergic neurons originating in the medial prefrontal cortex (mPFC) and hippocampus innervate dopaminergic neurons in the VTA causing an increase in dopaminergic neuronal activity and an increase in dopamine release in the nucleus accumbens (Tzschentke and Schmidt, 2003; Qi et al., 2009). This increased concentration of dopamine promotes glutamatergic pyramidal projection neurons in the mPFC to stay in a relatively depolarized state, thereby increasing the firing probability of the neurons (Tzschentke and Schmidt, 2003). This positive-feedback interaction between the glutamatergic and dopaminergic neurons results in a strengthening of the reward pathway in response to the rewarding stimuli (Jo et al., 1998; Tzschentke and Schmidt, 2003; Qi et al., 2009).
3.4 *C. elegans* as a Model Organism

Throughout this study, we use *Caenorhabditis elegans* (*C. elegans*) as our model organism. *C. elegans* are multicellular nematode organisms that are approximately 1mm in length. An individual *C. elegans* can be seen by the naked eye but they are mostly examined through the use of a microscope with a long confocal length to allow for more in depth analysis. *C. elegans* have a transparent cylindrical body that allows for many imaging techniques such as fluorescent microscopy, calcium imaging, and optogenetics to easily be used. *C. elegans* have two sexes, hermaphrodites and males. Hermaphrodites are capable of both self-fertilization and sexual reproduction and represent the majority of the population (Madl and Herman, 1979). The ability to asexually reproduce allows specific genetic manipulations conducted in *C. elegans* to easily be maintained through subsequent generations. In the lab, *C. elegans* are cultivated on Petri dishes containing agarose with a lawn of *E. coli* (OP50) that allows for many colonies to be maintained without difficulty. *C. elegans* have four distinct larval stages (L1-L4). The rate by which developmental and these specific larval stages occur can be controlled depending on the temperature of the incubator, and when food is scarce they are capable of going into a long-term hibernation stage called dauer (Lee and Kenyon, 2009). *C. elegans* were the first multicellular organisms to have their entire genome sequenced, and the developmental lineage of each of their cell types has been characterized. This allows them
to easily be genetically manipulated with exposure to chemicals and/or UV radiation (Chen et al., 2016).

### 3.5 GLT in *C. elegans*

![Diagram of the C. elegans Glutamate Synapse](image)

Many aspects of glutamatergic transmission are highly conserved between mammals and *C. elegans*, however key differences are present (de Bono and Maricq, 2005). As previously stated, in mammals, most synaptic glutamate is taken up by transporters located on the astrocytes that surround the synapse or on the postsynaptic membrane (Bergles and Jahr, 1997; Sheng and Kim, 2011). This tightly regulated activity ensures that glutamate excitotoxicity does not lead to cell death and that signal specificity remains intact (Kanai and Hediger, 2003). However, it is unclear how this process works for *C. elegans* since they do not have astrocytes, their GLT are not located on the postsynaptic neuron, and the glutamate synapse is under less stringent regulatory processes than found in mammals (Mano et al., 2007). In *C. elegans* there are six distinct GLT that have varying locations throughout the synapse: GLT-1, GLT-3, GLT-4, GLT-5, GLT-6, and GLT-7. GLT-1 is located on muscle cells that are located at a distance from the primary synapse and synaptic cleft, GLT-3 is located on canal
cells that are also located at a distance from the primary synapse and synaptic cleft and are primarily involved in metabolic processes, GLT-4 is located on the presynaptic neuron, GLT-5 is located within the pharyngeal region, most likely on canal cells, GLT-6 (not shown) is located on canal cells, and GLT-7 (not shown) is also located on canal cells but is primarily active only from the embryonic stage to the third stage of larval development (Mano et al., 2007). Throughout this study, all experimental procedures were completed on the wild type N2 strain along with C. elegans strains with varying glutamate transporter deletions: the first mutant strain has both GLT-1 and GLT-3 knocked out, the second mutant strain has GLT-4 knocked out, and the last mutant strain has GLT-5 knocked out.

3.6 C. elegans and GLT Research

GLT are functionally and structurally conserved between mammals and C. elegans (de Bono and Maricq, 2005). Furthermore, in C. elegans we are able to remove GLT without causing irreparable damage to the developmental processes of the organism. The deletion of GLT in higher order organisms, such as mammals, is more difficult as such action is often embryonically or developmentally lethal or results in severe neurodegeneration coupled with severe epileptic seizures (Rothstein et al., 1996). While there is no definitive proof to why such a discrepancy exists between mammals and C. elegans in terms of GLT modifications, it is likely that GLT cannot be knocked down in mammals because the glutamate synapse is much more tightly controlled as glutamate is the major excitatory neurotransmitter. In C. elegans glutamate is not the primary excitatory neurotransmitter but rather second to acetylcholine (Lewis et al., 1980; Schousboe, 1981). This most likely allows for the glutamate synapse to not be as tightly controlled and allows it to be manipulated in more extreme manners. Due to the widespread use of C. elegans in research, it is easy to obtain worms with different transporters missing and to grow colonies of multiple mutant worms. The behavior and memory capabilities of C. elegans can be effectively tested through the use of different behavioral assays and learning-paradigms (Murakami, 2007; Sasakura and Mori, 2013).

3.7 Research Questions

Although much research has been conducted to examine the different glutamate pathways that exist, the functional role of GLT in behavior, learning, and memory remains to be elucidated. In the Mano et al., 2007 paper, a group of behavioral paradigms were used to characterize the basic behavioral abilities in a multitude of GLT mutant strains and served as the starting point for our current set of experiments. Our study is aimed at examining the role of GLT on behavior, learning, and memory in C. elegans: Aim 1. Examining the differences in basic behaviors between different GLT mutants, Aim 2. Examining the differences in the ability to complete associative and nonassociative learning paradigms between different GLT mutants, and Aim 3. Examining
addiction as a type of memory and the ability for different mutant strains to express drug-seeking behavior and preference for known addictive drugs, ethanol and nicotine, in order to further characterize the role of individual GLT. To address these experimental questions, we used *C. elegans* strains that have specific GLT knocked-out and compared these mutants to the wild type N2 strain as well as to the other mutant strains. Basic behaviors tested include locomotion, response to a mechanosensory stimulus, response to the smell of an aversive chemical, and chemotaxis in response to a chemoattractant and a chemorepellent. Testing to see how glutamate transporters affect different learning and memory pathways including non-associative and associative learning paradigms was then conducted. Exposure to the addictive substances ethanol and nicotine was then examined as a type of memory. Furthermore, the ability for the different mutant strains to express drug-seeking behavior and initial preference for these drugs was evaluated. Through these experiments we hope to determine how transporter deletions and the specific transporter localizations impact the ability for a worm to perform basic behaviors, learn, remember, and exhibit properties of addiction.
4 Methods

4.1 Animals

*C. elegans* with varying GLT deletions were used throughout this study. The following mutant worm strains were obtained from the Caenorhabditis Genetics Center: glt-3(bz34) IV; glt-1(ok206) X, glt-4(bz69) II, and glt-5(bz70) II. Worms were raised in a 20°C incubator on 5 cm Petri dishes seeded with the OP50 strain of *Escherichia coli* (*E. coli*) to serve as the food source. The Petri dishes were chunked approximately every three days to maintain a fresh stock of worms. Chunking is the transfer of worms to a new Petri dish by removing a square of agar from the original Petri dish and placing it on the edge of the food source on the new Petri dish.

4.2 Basic Behaviors

4.2.1 Nose Touch

An individual L4-staged worm was transferred to an unseeded 5 cm Petri dish and allowed to acclimate to the new environment for approximately three minutes. An eyelash tool was then placed in front of the worm so that when the worm continued its forward trajectory it would come into contact with the tool. Whether a reversal occurred or not was recorded. Ten trials were completed for each worm with a 10-second resting period between trials.

4.2.2 Reversal Time

An individual L4-staged worm was transferred to an unseeded 5 cm Petri dish and allowed to acclimate to the new environment for approximately three minutes. The movement of the worm was then observed for two minutes and the amount of time the worm spent moving forward, moving in reverse, and remaining still was recorded.

4.2.3 Smell-on-a-Stick

An individual L4-staged worm was transferred to an unseeded 5 cm Petri dish and allowed to acclimate to the new environment for approximately three minutes. A toothpick dipped in a 30% octanol in ethanol solution was then placed in front of the worm. Whether a reversal occurred or not was recorded. Ten trials were completed for each worm with a 10-second resting period between trials.

4.2.4 Chemotaxis (Chemoattraction and Chemorepulsion)

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. In each of the quadrants, a dot was drawn that was equidistant from the center and from the other points and labeled alternately with T (for test) and with C (for control). A plate of worms was then washed with M9 (a mixed salt buffer) and centrifuged until a
worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of E. coli were present. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (double-deionized water) was then placed on the C dots. Finally, a 2 µl solution of equal amounts of 2% sodium azide and the test substance (either a 0.2% NaCl in double-deionized water or a 30% octanol in ethanol solution) was placed on the T dots (Iino and Yoshida, 2009). After 1 hour, the number of worms in each quadrant was recorded. The chemotactic index (CI), which ranges from 1 (complete chemoattraction) to -1 (complete chemorepulsion), was then calculated using the following equation:

Let $T_1 =$ The number of worms in the first test quadrant
Let $T_2 =$ The number of worms in the second test quadrant
Let $C_1 =$ The number of worms in the first control quadrant
Let $C_2 =$ The number of worms in the second control quadrant
Let $X = T_1 + T_2 + C_1 + C_2 =$ The total number of worms

$CI = \frac{(T_1 + T_2) - (C_1 + C_2)}{X}$

Figure 4: Chemotaxis Diagram

4.3 Associative Learning

4.3.1 Chemotaxis in Response to Butanone

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. In each of the quadrants, a dot was drawn that was equidistant from the center and from the other points and labeled alternately with T (for test) and with C (for control). A plate of
worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of *E. coli* were present. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (double-deionized water) was then placed on the C dots. Finally, a 2 µl solution of equal amounts of 2% sodium azide and a 30% butanone in ethanol solution was placed on the T dots. After 1 hour, the number of worms in each quadrant was recorded.

4.3.2 Association Control Paradigm

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. A dot was then drawn equidistant from the center and the edge of the Petri dish on the vertical line. One of the dots was labeled with a C (for control) and the other with a T (for test). A plate of worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of *E. coli* were present. The worms were then starved for exactly one hour in the microcentrifuge tube with M9 solution. The worms were then pipetted onto an unseeded 5 cm Petri dish and the lid streaked with 4 µl of a 30% butanone in ethanol solution. The plate was then covered in parafilm and left to condition undisturbed for exactly one hour. The worms on the conditioning plate were then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure consistency with association test paradigm. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (100% ethanol) was then placed on the C dot. Finally, a 2 µl solution of equal amounts of 2% sodium azide and a 30% butanone in ethanol solution was placed on the T dot. After 1 hour, the number of worms in each half of the Petri dish was recorded.

4.3.3 Association Paradigm

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. A dot was then drawn equidistant from the center and the edge of the Petri dish on the vertical line. One of the dots was labeled with a C (for control) and the other with a T (for test). A plate of worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then starved for exactly one hour in the microcentrifuge tube with M9 solution. The worms were then pipetted onto a seeded 5 cm Petri dish and the lid streaked with 4 µl of a 30% butanone in ethanol solution. The plate was then covered in parafilm and left to condition undisturbed for exactly one hour.
hour. The worms on the conditioning plate were then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of E. coli was present. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (100% ethanol) was then placed on the C dot. Finally, a 2 µl solution of equal amounts of 2% sodium azide and a 30% butanone in ethanol solution was placed on the T dot. After 1 hour, the number of worms in each half of the Petri dish was recorded.

4.4 Non-Associative Learning

4.4.1 Short-Term Tapping Habituation

A plate of worms two days post-chunking was placed on top of a mechanical tapper to enter the training phase of tapping habituation. The tapper was controlled via an arduino to tap the plate every 60 seconds for a total of 20 taps. After a 15-minute rest interval another trial of 20 taps began. A total of four trials were completed. After the training phase was complete, the plate of worms was left undisturbed for exactly one hour. The worms then entered the testing phase. The plate was tapped every 30 seconds for a total of ten taps. Each tap lasted 0.1 seconds and had a constant force of 1 N. Video was used to record the movement of the worms and used to analyze whether reversals occurred after each tap during the testing phase. Video analysis was conducted using the movement tracker, Bio-Track, on a virtual Ubuntu 12.04 hard drive. The software outputted BTF files that assigned an ID number to each worm and recorded the X- and Y-coordinates of the worm at each frame.
4.4.2 Long-Term Tapping Habituation (To be completed)

A plate of worms two days post-chunking will be placed on top of a mechanical tapper to enter the training phase of tapping habituation. The tapper will be controlled via an arduino to tap the plate every 60 seconds for a total of 20 taps. After a 15-minute rest interval another trial of 20 taps will begin. A total of four trials will be completed. After the training phase is complete, the plate of worms will be left undisturbed for exactly 24 hours. The worms will then enter the testing phase. The plate will be tapped every 30 seconds for a total of ten taps. Each tap will last 0.1 seconds and have a constant force of 1 N. Video will be used to record the movement of the worms and used to analyze whether reversals occurred after each tap during the testing phase. Video analysis will be conducted using the movement tracker, Bio-Track, on a virtual Ubuntu 12.04 hard drive. The software will output BTF files that will assign an ID number to each worm and record the X- and Y-coordinates of the worm at each frame.

4.5 Addiction Paradigms

4.5.1 Chemotaxis in Response to Ethanol and Nicotine

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. In each of the quadrants, a dot was drawn that was equidistant from the center and from the other points and labeled alternately with T (for test) and with C (for control). A plate of worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of \textit{E. coli} were present. Two \( \mu l \) of the worm pellet was then placed on the circle in the center of the plate. A 2 \( \mu l \) solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (double-deionized water) was then placed on the C dots. Finally, a 2 \( \mu l \) solution of equal amounts of 2% sodium azide and the test substance (either a 1.5 mM nicotine in ethanol solution or 100% ethanol) was placed on the T dots. After 1 hour, the number of worms in each quadrant was recorded.

4.5.2 Ethanol Association Paradigm

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. A dot was then drawn equidistant from the center and the edge of the Petri dish on the vertical line. One of the dots was labeled with a C (for control) and the other with a T (for test). A plate of worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of \textit{E. coli} was present. The worms were then starved for exactly one hour in the microcentrifuge tube. The worms were then pipetted onto an unseeded 5 cm Petri dish that contained 8 \( \mu l \) of ethanol and the lid streaked with 4 \( \mu l \) of a 30% butanone in ethanol solution. The plate was then covered in parafilm and left to condition
undisturbed for exactly one hour. The worms on the conditioning plate were then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of ethanol were present. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (double-deionized water) was then placed on the C dot. Finally, a 2 µl solution of equal amounts of 2% sodium azide and a 30% butanone in ethanol solution was placed on the T dot. After 1 hour, the number of worms in each half of the Petri dish was recorded.

4.5.3 Nicotine Association Paradigm

A 5 cm Petri dish was divided into four equal quadrants with a 0.5 cm radius circle drawn at the intersection point of the quadrants. A dot was then drawn equidistant from the center and the edge of the Petri dish on the vertical line. One of the dots was labeled with a C (for control) and the other with a T (for test). A plate of worms was then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of *E. coli* was present. The worms were then starved for exactly one hour in the microcentrifuge tube with M9 solution. The worms were then pipetted onto an unseeded 5 cm Petri dish that contained 8 µl of nicotine and the lid streaked with 4 µl of a 30% butanone in ethanol solution. The plate was then covered in parafilm and left to condition undisturbed for exactly one hour. The worms on the conditioning plate were then washed with M9 and centrifuged until a worm pellet formed at the bottom of the microcentrifuge tube. The worms were then washed two more times to ensure that no traces of nicotine were present. Two µl of the worm pellet was then placed on the circle in the center of the plate. A 2 µl solution of equal amounts of 2% sodium azide (to induce paralysis) and the control substance (double-deionized water) was then placed on the C dot. Finally, a 2 µl solution of equal amounts of 2% sodium azide and a 30% butanone in ethanol solution was placed on the T dot. After 1 hour, the number of worms in each half of the Petri dish was recorded.

4.5.4 Habituation to Nicotine (To be completed)

Worms will be placed on 5cm unseeded agar plates that contain nicotine at a 1.5 M concentration (the nicotine will be mixed into the agar before pouring). The spontaneous locomotion of the population of the worms on the plate (approximately 10 worms per plate) will be characterized at 10 minutes post exposure, 30 min post exposure, 45 min post exposure, 1 hour post exposure, 2 hours post exposure, and 3 hours post exposure. These results will then be compared to the spontaneous locomotion worms not exposed to plates containing nicotine. Video analysis will be conducted using the movement tracker, Bio-Track, on a virtual Ubuntu 12.04 hard drive. The software will output BTF files that will
assign an ID number to each worm and record the X- and Y-coordinates of the worm at each frame.

4.6 Statistical Analysis

All statistical analyses were performed in the free statistical software R.

4.6.1 Analysis of Basic Behaviors, Associative Learning, and Addiction Paradigms

The various groups within the respective paradigms were analyzed through the use of an ANOVA (form of linear regression). If the ANOVA output indicated that at least one group was significantly different from the others, a Tukey Honest Significant Difference (Tukey HSD) was then performed to see which mutant strain(s) was different from the wildtype strain. All data was plotted with lines indicating means.

4.6.2 Analysis of Non-Associative Learning Paradigm

Custom functions were written to combine csv (comma separated values) files and to calculate the velocity of each worm. Time was calculated by multiplying the time between frames (0.033 seconds) by the number of continuous frames that each worm was in. Distance was calculated using the distance formula, relying on the assumption that any given worm would travel in a straight line in the time between frames. The velocity for each individual worm was then calculated as the sum of all distance divided by the sum of all time for each worm. The average velocity for each strain and tap number was then calculated. Finally, the average velocity was standardized as relative to the average velocity of wildtype at the 10th tap (e.g. a velocity of 2 indicates that the worms were traveling twice as fast as the baseline velocity).

ANOVAs were performed across strains at certain taps (1, 4, 7, 10) and Tukey HSD was then performed to see which mutant strain(s) was different from the wildtype strain. ANOVAs were also performed within strains to see what velocities were different among taps and Tukey HSD was again performed to determine which taps differed within each respective strain. Tukey HSD adjusts for the fact that all pairwise comparisons are made, however a multiple comparison method must be used to adjust for the fact that throughout the analysis seven ANOVAs were performed. Therefore, a Bonferroni correction was used to compare the resulting p-values to the adjusted significance cutoff of $0.05/7 = 0.007$. All data was plotted with the line joining means at each tap for each strain.
5 Results

5.1 Basic Behaviors

5.1.1 Nose Touch

Nose touch is behavioral paradigm that tests the ability of a worm strain to sense and effectively respond to mechanosensory stimulation. When a wildtype worm comes into contact with the eyelash tool, it will likely reverse to avoid the object. In this paradigm, GLT-1; GLT-3 and GLT-4 mutants had an abnormal response to the mechanosensory stimulation and had a decreased number of reversals compared to wildtypes (Figure 6). Alternatively, GLT-5 mutants exhibited a normal response to the stimuli (Figure 6).

Figure 6: The Spontaneous Reversal Response to Mechanosensory Stimulation of Wildtype and GLT Mutant Strains. Whether a reversal occurred after mechanosensory stimulation with an eyelash tool was recorded for all strains. GLT-1; GLT-3 and GLT-4 mutants displayed a decreased number of reversals compared to wildtypes while GLT-5 exhibited a normal reversal response. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.
5.1.2 Reversal Time

Reversal time is a behavioral paradigm that tests the locomotion properties that a strain of worm possesses. Throughout this task, the spontaneous locomotion of the worms was categorized as moving forward, moving backwards, or remaining still. In comparison to the wildtype strain, all of our GLT mutant strains expressed decreased forward movement and increased periods of inactivity (Figure 7). GLT-1; GLT-3 and GLT-4 mutants additionally had increased backward movement while GLT-5 expressed unaltered reversal behavior (Figure 7).

![Graph showing Reversal Time](image)

Figure 7: The Reversal Behavior of Wildtype and GLT Mutant Strains. The locomotion of the worm strains was observed and categorized as moving forward, moving backwards, or remaining still. All GLT mutant strains expressed decreased forward movement and increased periods of inactivity while GLT-1; GLT-3 and GLT-4 mutants additionally had increased backward movement. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.

5.1.3 Smell-on-a-Stick

Smell on a stick is a behavioral paradigm that tests the ability for a worm strain to sense and effectively respond to an aversive olfactory stimuli. In response to
the aversive chemical octanol, a wildtype worm will reverse away from the source of the smell. Compared to wildtypes, GLT-1; GLT-3 and GLT-5 mutants were deficient and expressed a decrease in reversal response (Figure 8). However, GLT-4 mutants expressed a normal reversal in response to octanol (Figure 8).

Figure 8: The Reversal Response to An Aversive Chemical Stimuli of Wildtype and GLT Mutant Strains. Whether a reversal occurred after the introduction of octanol, an aversive chemical, was recorded for all strains. GLT-1; GLT-3 and GLT-5 mutants expressed a decrease in reversal response compared to wildtypes while GLT-4 expressed a normal reversal response. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.

5.1.4 Chemotaxis (Chemoattraction and Chemorepulsion)

Chemoattraction is a behavioral paradigm is used to test the ability of a worm strain to preferentially move towards a known chemoattractant. Wildtype worms preferentially move towards to a 0.2% NaCl solution with a chemotactic index of approximately 0.3 (Figure 9). In contrast, all GLT mutant strains showcased decreased chemoattraction and moved towards the NaCl solution to a lesser degree with a chemotactic index around 0.1 (Figure 9).

Chemorepulsion is a behavioral paradigm is used to test the ability of a worm
strain to preferentially move away from a known chemorepellent. Wildtype worms preferentially move away from an octanol solution with a chemotactic index of approximately -0.25 (Figure 9). GLT-4 mutants expressed an increased chemotactic index, which shows that they have a decreased chemorepulsion response to octanol compared to wildtypes (Figure 9). GLT-1; GLT-3 mutants expressed unaltered chemorepulsion. Interestingly, GLT-5 mutants expressed a hyperactive chemorepulsion response and had a chemotactic index of around -0.4 (Figure 9).

Figure 9: Chemotaxis Ability of Wildtype and GLT Mutant Strains. The chemotactic index in response to either an attractive or repulsive chemical stimuli was calculated for each strain. Compared to wildtypes, all GLT mutant strains expressed decreased chemoattraction to NaCl. GLT-4 mutants expressed decreased chemorepulsion to octanol. GLT-1; GLT-3 mutants expressed unaltered chemorepulsion. GLT-5 mutants express a hyperactive chemorepulsion response to octanol. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.
5.2 Associative Learning

5.2.1 Association Control Paradigm

This behavioral paradigm is used to test the ability of a worm strain to preferentially move away from a known chemorepellent, butanone, and whether an association period with only butanone is enough to cause decreased chemorepulsion. This is a control to make sure that the worms are simply not habituating to the butanone over time and need a conditioning period with a positive stimulus to result in chemoattractant behavior. Before the association trial, wildtype worms preferentially move away from a butanone solution with a chemotactic index of approximately -0.28 (Figure 10). GLT-4 and GLT-5 mutants expressed an increased chemotactic index, which shows they had a decreased chemorepulsion response to butanone compared to wildtypes (Figure 10). GLT-1; GLT-3 mutants expressed unaltered chemorepulsion (Figure 10). After the association trial, wildtype worms preferentially move away from a butanone solution but to a significantly decreased degree compared to the pre-association trial. All mutant strains expressed an increased chemotactic index compared to wildtypes (Figure 10).
Figure 10: Chemotactic Response to Butanone Pre- and Post-Association Without a Rewarding Stimulus. The chemotactic index in response to butanone pre- and post- association was calculated for each strain. In the pre-association trial, GLT-4 and GLT-5 mutants expressed a decreased chemorepulsion response to butanone while GLT-1; GLT-3 mutants expressed unaltered chemorepulsion in comparison to wildtypes. In the post-association trial, wildtypes expressed decreased chemorepulsion in comparison to the pre-association trial. All mutant strains expressed a decreased chemorepulsion response compared to wildtypes. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.

5.2.2 Association Paradigm

This behavioral paradigm is used to test the ability of a worm strain to preferentially move towards a known chemorepellent, butanone, after an association period with the food source *E. coli*. After the association period, wildtype worms expressed a chemotactic index with the opposite sign that showcases a learned chemoattraction to the butanone solution (Figure 11). GLT-1; GLT-3 mutants expressed unaltered learned chemoattraction compared to wildtypes (Figure 11). GLT-4 and GLT-5 mutants expressed a decreased chemotactic index, which shows they have a decreased chemoattraction response to butanone compared to wildtypes (Figure 11).
Figure 11: Chemotactic Response to Butanone Post-Association With *E. coli*. The chemotactic index in response to butanone post-association was calculated for each strain. After the association period, wildtype worms expressed chemoattraction towards butanone. Compared to wildtypes, GLT-1:GLT-3 mutants expressed unaltered learned chemoattraction while GLT-4 and GLT-5 mutants expressed decreased chemoattraction. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.
5.3 Non-Associative Learning

5.3.1 Short-Term Tapping Habituation

This behavioral paradigm is used to test the ability of a worm strain to habituate to repeated mechanical tapping stimulation. The GLT-4 mutant strain was not analyzed for this paradigm due to an abnormal initial response, continued forward movement to tapping, observed during the qualitative analysis performed previously. At the first tap in the testing phase, wildtype worms responded with movement of a significantly higher velocity than all mutant strains (Figure 12 A). For the taps two through nine, all strains responded with the same level of movement (Figure 12 A). At the tenth and final tap of the testing phase, wildtype worms responded with a significantly decreased velocity than GLT-1; GLT-3 mutants while GLT-5 mutants expressed unaltered responsive movement relative to wildtypes (Figure 12 A). The velocity of the worms were then analyzed within strains. Wildtype worms had a significantly higher velocity at the first tap compared to all subsequent taps (Figure 12 B). At the tenth tap, wildtypes had a significantly lower velocity than at taps one, two, five, six, seven and eight (Figure 12 B). Both GLT-1; GLT-3 and GLT-5 strains expressed unaltered velocity throughout the tapping paradigm (Figure 12 C and D).
Figure 12: **Habituation in Response to a Tapping Stimulus.** The velocity of the worms immediately after each tap in the testing phase was calculated and standardized relative to the velocity of the wildtype strain at the final tap. At the first tap, all GLT mutant strains expressed a decrease in velocity compared to wildtypes (A). For the taps two through nine, all strains responded with the same level of movement (A). At the tenth and final tap, GLT-1; GLT-3 mutants expressed an increase in velocity while GLT-5 mutants expressed unaltered movement in comparison to wildtypes (A). Wildtype worms had a significantly higher velocity at the first tap compared to all subsequent taps (B). Within strain analysis were then completed. At the tenth tap, wildtypes had a significantly lower velocity than at taps one, two, five, six, seven and eight (B). Both GLT-1; GLT-3 and GLT-5 strains expressed unaltered velocity throughout the paradigm (C and D). * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis and Bonferroni correction to account for multiple comparisons.
5.4 Addiction Paradigms

5.4.1 Ethanol Chemotaxis

This behavioral paradigm is used to test whether ethanol acts as a chemoattractant to different worm strains. Wildtypes preferentially moved towards ethanol with a chemotactic index of approximately 0.11 (Figure 13). In contrast, all GLT mutant strains showcased decreased chemoattraction and moved towards ethanol to a lesser degree (Figure 13).

![Chemotactic Response to Ethanol](image)

Figure 13: **Chemotactic Response to Ethanol.** The chemotactic index in response to ethanol was calculated for each strain. Compared to wildtypes, all mutant strains expressed decreased chemoattraction to ethanol. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.

5.4.2 Ethanol Associative Learning

This behavioral paradigm is used to test the ability of a worm strain to preferentially move towards a known chemorepellent, butanone, after an association period with ethanol. After the association period, wildtype worms expressed a chemotactic index with the opposite sign that showcases a learned chemotraction to the butanone solution (Figure 14). All mutant strains expressed
A decreased chemotactic index, which shows they have a decreased chemoattraction response to butanone compared to wildtypes after ethanol association (Figure 14).

Figure 14: Chemotactic Response to Butanone Post-Association With Ethanol. The chemotactic index in response to butanone post-association was calculated for each strain. After the association period, wildtype worms expressed chemoattraction towards butanone. All mutant strains expressed decreased learned chemoattraction to ethanol. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.

5.4.3 Nicotine Chemotaxis

This behavioral paradigm is used to test whether nicotine acts as a chemoattractant to different worm strains. Wildtypes preferentially moved towards a nicotine solution with a chemotactic index of approximately 0.10 (Figure 15). All GLT mutants expressed unaltered chemoattraction to the nicotine solution (Figure 15).
Figure 15: **Chemotactic Response to Nicotine.** The chemotactic index in response to nicotine was calculated for each strain. Compared to wildtypes, all mutant strains expressed unaltered chemoattraction to nicotine. * denotes a p-value of $< 0.05$, ANOVA with a Tukey HSD post-hoc analysis.

### 5.4.4 Nicotine Associative Learning

This behavioral paradigm is used to test the ability of a worm strain to preferentially move towards a known chemorepellent, butanone, after an association period with nicotine (Figure 16). All mutant strains expressed a decreased chemotactic index, which shows they have a decreased chemoattraction response to butanone compared to wildtypes after nicotine association (Figure 16).
Figure 16: **Chemotactic Response to Butanone Post-Association With Nicotine.** The chemotactic index in response to butanone post-association was calculated for each strain. After the association period, wildtype worms expressed chemoattraction towards butanone. All mutant strains expressed decreased learned chemoattraction to nicotine. * denotes a p-value of < 0.05, ANOVA with a Tukey HSD post-hoc analysis.
6 Discussion

6.1 Part 1: Implications of GLT Deletions on Basic Behaviors

The first section of this study sought to replicate and supplement behavioral paradigms previously studied in *C. elegans* with specific GLT deletions. Throughout this study, three mutant strains with varying GLT deletions encompassing GLT-1, GLT-3, GLT-4, and GLT-5 were compared to the wildtype N2 strain. In order to investigate the role of GLT in basic behaviors in *C. elegans*, we modified four distinct behavioral paradigms found in Mano et al., 2007. These four tasks include: nose touch, reversal time, smell on a stick, and chemotaxis and were aimed at testing the ability of the different strains to sense and respond to mechanosensory and chemical stimuli.

Throughout our study, we obtained basic behavior results that are similar in nature but not identical to those reported in previous studies. For the nose touch paradigm, which tests the ability of the worms to sense a mechanosensory stimulus and respond with a reversal, we observed both the GLT-1; GLT-3 and GLT-4 mutants as being deficient in the percentage of the mechanosensory stimuli that resulted in a reversal response. However, the GLT-5 mutant strain expressed an unaltered reversal response to the stimulus. Previously, Mano et al., 2007 had shown that both GLT-4 and GLT-5 mutant strains are deficient in this sensory task, but do not provide results for the GLT-1; GLT-3 mutants.

For the reversal time paradigm, which categorizes the spontaneous movement, we observed all mutant strains exhibiting decreased spontaneous forward movement and increased periods of inactivity. Furthermore, both GLT-1; GLT-3 and GLT-4 mutants expressed increased spontaneous backward movement when compared to the wildtype strain. Prior research on GLT mutants have only analyzed the forward and backward movement of the different worm strains (Mano et al., 2007). In those experiments it was observed that both GLT-1; GLT-3 and GLT-4 have unaltered spontaneous movement while the GLT-5 mutant strain was not analyzed (Mano et al., 2007).

Through the smell on a stick paradigm, which is used to test the ability of the worm strains to sense an aversive chemical stimuli and exhibit a reversal response away from the chemical, we observed reversal deficits for the GLT-1; GLT-3 and GLT-5 mutant strains while the GLT-4 strain did not have such a deficit. Previously, the time between exposure to the aversive chemical stimuli and a reversal response was recorded rather than whether or not a reversal response occurred. In that paradigm, it was shown that GLT-4 mutants exhibited an increased delay between the introduction of the stimulus and a reversal response, and GLT-5 mutants had an unaltered response delay while GLT-1; GLT-3 mutant strain was not analyzed (Mano et al., 2007). We modified our behavioral paradigm to record whether an appropriate response occurred without distinction between the time delay exhibited between the stimulus exposure and onset of a reversal. These modifications were introduced to limit the impact that the altered movement ability that the mutants strains previously exhibited
had on the paradigm and to focus the paradigm solely on chemical stimuli detection and response.

The final basic behavior paradigm tested was chemotaxis, which tests the ability of the worm strains to express chemoattraction and chemorepulsion behaviors. Throughout this paradigm, we observed that all mutant strains exhibited decreased chemoattraction to a 0.2% NaCl solution. However, only the GLT-4 mutant strain exhibited decreased chemorepulsion away from a 30% octanol in ethanol solution, the GLT-1; GLT-3 strain exhibited unaltered chemorepulsion, and GLT-5 mutants exhibited a hyperactive chemotactic response to the octanol solution. These results suggest that chemoattraction relies more on GLT than chemorepulsion does. Previous paradigms used on GLT mutants tested only chemoattraction and used an isoamyl alcohol solution (Mano et al., 2007). Through this paradigm it was seen that GLT-4 mutants exhibited decreased chemoattraction and GLT-5 mutants exhibited unaltered chemoattraction while GLT-1; GLT-3 mutants were not analyzed (Mano et al., 2007).

Our results indicate that different sensory systems rely on GLT differently and that even with deletion of multiple GLT subtypes proper sensory detection and response is possible. Throughout this section of the study, we were mostly able to replicate and fine-tune previous findings reported in the literature, however, these experiments served as a baseline for the development and analysis of our learning, memory, and addiction paradigms.

6.2 Part 2: Implications of GLT Deletions on Associative Learning

In the second section of our study, we sought to elucidate the role of GLT in the learning and memory pathways specifically involved in associative learning. Prior research has suggested a role for glutamatergic pathways in associative learning for *C. elegans* but only has evidence linking NMDA-type ionotropic glutamate receptors being involved and not GLT (Kano et al., 2008; Kauffman et al., 2011; Lau et al., 2013; Pereira and van der Kooy, 2013). However, GLT control the amount of free-floating glutamate present in the synapse, which directly impacts the functionality of NMDA receptors, and are likely to impact associative memory. Our present study was aimed at determining if deleting specific GLT led to altered or deficient associative learning capabilities.

Previous studies have shown that the OP50 strain of *E. coli*, which is the *C. elegans* primary food source, is capable of acting as a conditioning stimulus in associative learning paradigms (Amano and Maruyama, 2011; Ardiel and Rankin, 2010; Cho et al., 2016; Pereira and van der Kooy, 2012; Sasakura and Mori, 2013; Stein and Murphy 2014). Therefore, our associative learning paradigm utilized *E. coli* as the conditioning stimulus and paired the food with a butanone in ethanol solution, a known chemorepellent (Kauffman et al., 2011). In a preliminary study, we tested the degree of chemorepulsion away from the butanone solution in unconditioned worms. Our data shows that all mutant strains exhibit butanone chemorepulsion but GLT-4 and GLT-5 mutants express an increased chemotactic index compared to wildtypes, which indicates a
decreased aversion to the stimulus. Throughout the association paradigm, we used the butanone solution as the chemorepellent instead of the octanol solution previously used in the chemotaxis paradigm because butanone is not as strong of a chemorepellent and therefore requires a shorter conditioning period (Kauffman et al., 2011).

In wildtypes, after performing the associative learning paradigm, we saw similar results as previously reported with wildtypes expressing a chemotactic sign reversal resulting in chemoattraction toward a chemorepellent after \textit{E. coli} conditioning (Kano et al., 2008; Kauffman et al., 2011; Lau et al., 2013; Pereira and van der Kooy, 2013). All mutant strains exhibited associative learning and a corresponding chemotactic sign reversal. GLT-1; GLT-3 mutants express unaltered association while GLT-4 and GLT-5 mutants express decreased chemoattraction toward the butanone solution in the post-association period compared to wildtypes. However, the deficits seen in chemoattraction in the post-association period cannot be directly attributed to deficits in learning ability and may in fact be attributable to deficits previously observed in chemotaxis and spontaneous movement ability. In order to showcase that a conditioning stimulus and not just an association period is necessary for a chemotactic sign reversal, we performed an associative learning control paradigm that followed the same procedure but did not include \textit{E. coli} during the association period. With this paradigm, the wildtype and mutant strains exhibited decreased chemorepulsion in the post-association period but did not exhibit chemoattraction or associative learning.

6.3 Part 3: Implications of GLT Deletions on Non-Associative Learning

In the third section of our study, we sought to elucidate the role of GLT in the learning and memory pathways specifically involved in non-associative learning. Prior research has also implicated the glutamatergic pathway and specifically NMDA-type ionotropic glutamate receptors in non-associative learning for \textit{C. elegans} (Lau et al., 2013; Pereira and van der Kooy, 2013; Rose et al., 2002). Our present study was aimed at determining if deleting specific GLT led to altered or deficient non-associative learning capabilities.

Previous studies have shown that habituation to a mechanosensory stimulus, such as tapping, is an effective mechanism to test non-associative abilities in varying \textit{C. elegans} strains (Rankin, 2000; Rankin et al., 2009; Rose et al., 2002). Therefore, for our non-associative learning paradigm we utilized mechanosensory stimulation in the form of tapping to observe habituation behavior in our GLT mutant strains in comparison to the wildtype strain. Our paradigm was completed through the use of an automated mechanical tapper that was electrically controlled by an Arduino microcontroller board. The tapping paradigm used is a modified form of the procedure developed by Rose et al., 2002 and results in a similar habituation outcome in the wildtype strain. Preliminary experiments indicated that GLT-4 mutants did not reverse in response to mechanical tapping stimulation and therefore are not analyzed in the present ex-
experiment. After between-strain analysis was performed, differences were seen between strains at the first and tenth tap. At the first tap, GLT-1; GLT-3 and GLT-5 mutant strains both expressed decreased velocity in comparison to wildtypes after the tap occurred, but at the tenth tap only GLT-1; GLT-3 differed in velocity from the wildtype strain. Within-strain analysis was also performed and showed that as the taps progressed wildtype was the only strain that had a consistent decrease in velocity that could be attributed to habituation and therefore is the only strain that appears to exhibit proper non-associative learning. However, preliminary analysis performed without the use of the automated mechanical tapper suggested that GLT-1; GLT-3 mutants were capable of habituating to tapping. The qualitative data was obtained from individual worms while the quantitative data examined the impact of tapping on 20 to 40 worms at once. This difference in trial size and subsequent interaction between worms in the latter could cause the results to have to be interpreted differently. Further experiments are needed to isolate the true non-associative abilities of the mutant strains and to elucidate the role of GLT in non-associative learning.

6.4 Part 4: Implications of GLT Deletions on Addiction

The fourth section of this study sought to determine the role in GLT in the formation of addictive memories and the resulting display of drug-seeking behavior. Previous research has indicated that addiction is a subtype of long-term episodic memory that results in cellular and molecular neural correlates throughout the memory centers of the brain (Berke and Hyman, 2000; Boning, 2009; Kauer, 2004; Nestler, 2013; Volkow et al., 2002). Furthermore, multiple behaviors and molecular processes associated with addiction have been observed in C. elegans (Engelman et al., 2016; Feng et al., 2006; Schafer, 2004; Sellings et al., 2013; Ward et al., 2009; Wolf and Heberlein, 2003). Our present study was aimed at determining if deleting specific GLT led to altered or deficient initial preference and drug-seeking behavior toward two known addictive drugs: ethanol and nicotine.

In order to test whether an innate preference exists for these two drugs, a chemotaxis assay was completed with ethanol and nicotine as the test substances in their respective trials. Wildtype worms exhibited slight chemoattraction toward ethanol across multiple chemotaxis trials. All GLT mutant strains also exhibited chemoattraction but to a lesser degree than that present in wildtypes. All strains additionally expressed slight chemoattraction toward a nicotine in ethanol solution. These results indicate that without prior exposure or conditioning, all of the strains used in this study express an innate preference for these drugs at the concentrations used. Furthermore, this suggests that ethanol and nicotine detection and preference may be controlled by different molecular processes with ethanol processes relying more on unaltered GLT functioning. This conclusion is supported by the fact that ethanol and nicotine have two distinct mechanisms of action in the nervous system. Ethanol mainly interacts with the GABAergic pathway as an agonist for the $GABA_A$ receptor, but has also been shown to have an antagonistic effect on the glutamatergic pathway via
NMDA receptors (Wright et al., 1996). Nicotine primarily acts as an agonist at nicotinic cholinergic receptors and has not been shown to directly impact the glutamatergic pathway (Benowitz, 2009).

A modified associative learning paradigm, of novel design, was then used to determine if the different worm strains would exhibit a type of controlled drug-seeking behavior toward ethanol and/or nicotine. In these experiments, E. coli was replaced with either ethanol or nicotine as the conditioning stimulus. In wildtypes, after performing the modified associative learning paradigm, we saw similar results as previously seen with wildtypes expressing a chemotactic sign reversal resulting in chemoattraction toward a chemorepellent after conditioning with either ethanol or nicotine. All mutant strains exhibited associative learning and a corresponding chemotactic sign reversal for both drugs but expressed decreased chemoattraction toward the butanone solution in the post-association periods compared to wildtypes in each respective trial. However, the deficits seen in chemoattraction in the post-association period again cannot be directly attributed to deficits in learning ability and may in fact be attributable to deficits previously observed in chemotaxis and spontaneous movement ability. Interestingly, the chemotactic indices seen in the post-conditioning period are of a significantly larger magnitude than those observed in the innate preference trials for either drug. This suggests that with long-term exposure the innate preference for the two drugs increases and results in a more intense chemoattraction to the addictive substances. The post-conditioning chemotactic indices for both drugs are also in the same magnitude as those seen in the post-conditioning trials where E. coli was used as the conditioning stimulus. In addition to showing that ethanol and nicotine both provide the same positive reward in association paradigms as food, these results also show that over a relatively short conditioning period all worm strains begin to express drastic and reproducible drug-seeking behavior.

6.5 Conclusions

Together our results from the basic behavior, associative learning, non-associative learning, and addiction paradigms suggest that basic behaviors may rely on different GLT than learning and memory do. Furthermore, the data indicate that the differences seen between different mutant strains may be due to the localization of the specific GLT within the C. elegans nervous system. Across our behavioral paradigms it is seen that GLT-4 mutants express the greatest observable deficits. Moreover, research from other projects within the Bauer Lab indicate that GLT-4 mutants have a significantly decreased lifespan compared to the wildtype strain. As noted previously, GLT-4 is the only GLT located directly in the glutamate synapse while the other other five GLT found in C. elegans are located at a distance away from the synapse (Mano et al., 2007). While we have no direct evidence to determine if and how the specific localization of GLT-4 has a greater impact on behavior, we hypothesize that the loss of GLT-4 may result in an increase in excitotoxicity and subsequent increase in neuronal cell death (Lau and Tymianski, 2010; Mano and Driscoll, 2009). As
the other GLT are not located directly at the synapse it remains a likely possibility that they contribute less to preventing excitotoxicity and have a greater involvement in glutamate metabolism and excretion.

6.6 Future Directions

Further experimentation and analysis is needed to elucidate how GLT differentially affects behavior, learning, memory, and addiction and how localization impacts these effects in *C. elegans*. As our present study only looks at the impacts of GLT-1; GLT-3, GLT-4, and GLT-5 deletions, to determine the role of each GLT we would need to perform our basic behavior, learning and memory, and addiction paradigms on both GLT-1 and GLT-3 single mutants as well as GLT-6 mutants.

Our associative learning experiments showcase that all of the mutant strains are capable of possessing associative learning but fail to determine if their ability to maintain that specific association lasts to further time points. Therefore, it is necessary to expand the associative learning paradigm to see how long associative memory lasts in wildtypes and how the GLT mutant strains compare. The non-associative learning paradigm also fails to look at if the formation of long-term memory is different between the strains. However, our training and testing phases for the tapping paradigm could be modified to be completed over a 24 hour period in order to test the ability for the strains to exhibit non-associative long-term memory. We would also like to examine the impact of GLT deletion on thermodaxis and thermomemory, which is another type of associative learning that tests the ability for the worms to associate a feeding status, for example being well-fed or starving, with a particular temperature and move towards that temperature when it is associated with being well-fed or away from that temperature when it is associated with starvation when a temperature gradient is present.

Although our studies provide results on initial drug preference and drug seeking behavior they fail to provide evidence for the many other processes associated with addiction such as the formation of tolerance. We have preliminary data that showcases specific time points for key changes in response to these drugs and the formation of tolerance for wildtype strains. However, these time points need to be elucidated for mutant strains before video analysis software can be used to quantitatively evaluate ethanol and nicotine tolerance in our worm strains. Furthermore, we would like to examine these innate preference and drug-seeking behavior paradigms with drugs that act on other various neurotransmitter systems. We are particularly interested in looking at both ketamine, which acts directly on the glutamate system as an NMDA receptor antagonist, and cocaine, which modulates the uptake of dopamine and other monoamine neurotransmitters.

Throughout this study we also experienced a multitude of technological challenges that limited the scope and power of the paradigms we developed. For example, our present non-associative learning set-up allows for only a small portion of the Petri dish to be recorded throughout the duration of the paradigm.
A new tapping set-up would preferentially include a recording technology setup that allowed us to record the entirety of the plate and then use our same video analysis software to analyze the worm movements in response to the mechanical stimuli.
7 Literature Cited


8 Appendix

8.1 Data Visualization and Analysis for Basic Behaviors, Associative Learning, and Addiction (R Code)

colors<- c("violetred4", "dodgerblue4", "green4", "darkgoldenrod3")

# Read in nosepoke csv file
nosepoke <- read.csv(file="CSV Files/Nose Poke Thesis.csv", header=TRUE, stringsAsFactors=FALSE)
lm_nose_reverse <- lm(Reversal~Strain, data=nosepoke)
anova_nose_reverse <- aov(lm_nose_reverse)
TukeyHSD(anova_nose_reverse)

# Subsets nose poke - reversal
wildtype_nose_reversal_subset <- nosepoke$Reversal[nosepoke$Strain=="wildtype"]
Glt3.Glt1_nose_reversal_subset <- nosepoke$Reversal[nosepoke$Strain=="Glt-3, Glt-1"]
Glt4_nose_reversal_subset <- nosepoke$Reversal[nosepoke$Strain=="Glt-4"]
Glt5_nose_reversal_subset <- nosepoke$Reversal[nosepoke$Strain=="Glt-5"]

# Plots the nose poke reversal
par(las=1)
plot(seq(0.8, 1.2, length.out=30), wildtype_nose_reversal_subset, main="",
col=colors[1], pch=16, xlim=c(0.5,4.5), ylim=c(0,10), xaxt="n", xlab="",
ylab="Number of Reversals")axis(1, at=1:4, labels=rep("",4))
text(x = seq(1, 4, by=1), par("usr")[3] - 1.2, labels = c("Wildtype", "* Glt-1; Glt-3",
"* Glt-4", "Glt-5"), srt = 45, pos = 1, xpd = TRUE)

points(seq(1.8, 2.2, length.out=30), Glt3.Glt1_nose_reversal_subset,
col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=30), Glt4_nose_reversal_subset,
col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=30), Glt5_nose_reversal_subset,
col=colors[4], pch=16)

points(c(0.7, 1.3), rep(mean(wildtype_nose_reversal_subset),2),
type="l", lwd=2, col=colors[1])
points(c(1.7, 2.3), rep(mean(Glt3.Glt1_nose_reversal_subset),2),
type="l", lwd=2, col=colors[2])
points(c(2.7, 3.3), rep(mean(Glt4_nose_reversal_subset),2),
type="l", lwd=2, col=colors[3])
points(c(3.7, 4.3), rep(mean(Glt5_nose_reversal_subset),2),
type="l", lwd=2, col=colors[4])

# # # # # # # # # # # # # # # # # # # # # # # # # #
# Read in smell on a stick csv file
smellonastick <- read.csv(file="CSV Files/Smell on a Stick Thesis.csv", header=TRUE, stringsAsFactors=FALSE)
lm_smell_reverse <- lm(Reversal~Strain, data=smellonastick)
aova_smell_reverse <- aov(lm_smell_reverse)
TukeyHSD(aova_smell_reverse)

# Subsets nose poke - reversal
wildtype_smell_reversal_subset <- smellonastick$Reversal[smellonastick$Strain=="wildtype"]
Glt3.Glt1_smell_reversal_subset <- smellonastick$Reversal[smellonastick$Strain=="Glt-3; glt-1"]
Glt4_smell_reversal_subset <- smellonastick$Reversal[smellonastick$Strain=="Glt-4"]
Glt5_smell_reversal_subset <- smellonastick$Reversal[smellonastick$Strain=="Glt-5"]

# Plots the smell on a stick reversal
par(las=1)
plot(seq(0.8, 1.2, length.out=30), wildtype_smell_reversal_subset, main="", col=colors[1], pch=16, xlim=c(0.5,4.5), xlab="Number of Reversals") text(x = seq(1, 4, by=1), par("usr")[3] - 1.2, labels = c("Wildtype", "* Glt-1; Glt-3", "Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
points(seq(1.8, 2.2, length.out=30), Glt3.Glt1_smell_reversal_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=30), Glt4_smell_reversal_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=30), Glt5_smell_reversal_subset, col=colors[4], pch=16)

# Read in reversal time csv file
reversaltimetime <- read.csv(file="CSV Files/Reversal Time Thesis.csv", header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_reversaltime_forwards <- lm(Forward~Strain, data=reversaltime)
lm_reversaltime_backwards <- lm(Backwards~Strain, data=reversaltime)
lm_reversaltime_nomovement <- lm(No.Movement~Strain, data=reversaltime)

# ANOVA
anova_reversaltime_forwards <- aov(lm_reversaltime_forwards)
anova_reversaltime_backwards <- aov(lm_reversaltime_backwards)
anova_reversaltime_nomovement <- aov(lm_reversaltime_nomovement)

summary(anova_reversaltime_forwards)
summary(anova_reversaltime_backwards)
summary(anova_reversaltime_nomovement)

# Post-Hoc Tukey Test
TukeyHSD(anova_reversaltime_forwards)
TukeyHSD(anova_reversaltime_backwards)
TukeyHSD(anova_reversaltime_nomovement)

# Averages and standard deviation for reversal - forwards
wildtype_reversal_forwards_subset <- reversaltime$Forward[reversaltime$Strain=="wildtype"]
Glt3.Glt1_reversal_forwards_subset <- reversaltime$Forward[reversaltime$Strain=="Glt-3; glt-1"]
Glt4_reversal_forwards_subset <- reversaltime$Forward[reversaltime$Strain=="Glt-4"]
Glt5_reversal_forwards_subset <- reversaltime$Forward[reversaltime$Strain=="Glt-5"]

# Averages and standard deviation for reversal - backwards
wildtype_reversal_backwards_subset <- reversaltime$Backwards[reversaltime$Strain=="wildtype"]
Glt3.Glt1_reversal_backwards_subset <- reversaltime$Backwards[reversaltime$Strain=="Glt-3; glt-1"]
Glt4_reversal_backwards_subset <- reversaltime$Backwards[reversaltime$Strain=="Glt-4"]
Glt5_reversal_backwards_subset <- reversaltime$Backwards[reversaltime$Strain=="Glt-5"]

# Averages and standard deviation for reversal - no movement
wildtype_reversal_no.movement_subset
<- reversaltime$No.Movement[reversaltime$Strain=="wildtype"]
Glt3.Glt1_reversal_no.movement_subset
<- reversaltime$No.Movement[reversaltime$Strain=="Glt-3; glt-1"]
Glt4_reversal_no.movement_subset
<- reversaltime$No.Movement[reversaltime$Strain=="Glt-4"]
Glt5_reversal_no.movement_subset
<- reversaltime$No.Movement[reversaltime$Strain=="Glt-5"]

# Plots spontaneous movement
par(las=1)
plot(seq(0.8, 1.2, length.out=50), wildtype_reversal_forwards_subset, main="",
col=colors[1], pch=16, xlim=c(0.5,14.5), ylim=c(0,120), xaxt="n", xlab="", ylab="Seconds")
axis(1, at=c(1:4, 6:9, 11:14), labels=rep("",12))
text(x = seq(1-0.5, 14-0.5, by=1), par("usr")[3] - 20, labels = c("Wildtype", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5", "", "Wildtype", "* Glt-1; Glt-3", "* Glt-4", "Glt-5", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
axis(3, at=c(2.5,7.5,12.5), labels=c("Forwards", "Backwards", "No Movement"))
points(seq(1.8, 2.2, length.out=50), Glt3.Glt1_reversal_forwards_subset,
col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=50), Glt4_reversal_forwards_subset,
col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=50), Glt5_reversal_forwards_subset,
col=colors[4], pch=16)
points(c(0.5, 1.5), rep(mean(wildtype_reversal_forwards_subset),2),
type="l", lwd=2, col=colors[1])
points(c(1.5, 2.5), rep(mean(Glt3.Glt1_reversal_forwards_subset),2),
type="l", lwd=2, col=colors[2])
points(c(2.5, 3.5), rep(mean(Glt4_reversal_forwards_subset),2),
type="l", lwd=2, col=colors[3])
points(c(3.5, 4.5), rep(mean(Glt5_reversal_forwards_subset),2),
type="l", lwd=2, col=colors[4])

points(seq(5.8, 6.2, length.out=50), wildtype_reversal_backwards_subset,
col=colors[1], pch=16)
points(seq(6.8, 7.2, length.out=50), Glt3.Glt1_reversal_backwards_subset,
col=colors[2], pch=16)
points(seq(7.8, 8.2, length.out=50), Glt4_reversal_backwards_subset,
col=colors[3], pch=16)
points(seq(8.8, 9.2, length.out=50), Glt5_reversal_backwards_subset,
col=colors[4], pch=16)
points(c(5.5, 6.5), rep(mean(wildtype_reversal_backwards_subset),2),

48
Read in chemotaxis csv file
chemotaxis <- read.csv(file="CSV Files/Chemotaxis Thesis.csv", header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_oct <- lm(Octanol.Index~Strain, data=chemotaxis)
lm_nacl <- lm(NaCl.Index~Strain, data=chemotaxis)

# ANOVA
anova_oct <- aov(lm_oct)
anova_nacl <- aov(lm_nacl)

summary(anova_oct)
summary(anova_nacl)

# Post-Hoc Tukey Test
TukeyHSD(anova_oct)
TukeyHSD(anova_nacl)

# Averages and standard deviation for chemotaxis - Octanol
wildtype_octanol_subset <- chemotaxis$Octanol.Index[chemotaxis$Strain=="wildtype"]
Glt3.Glt1_octanol_subset <- chemotaxis$Octanol.Index[chemotaxis$Strain=="Glt-3; Glt-1"]
Glt4_octanol_subset <- chemotaxis$Octanol.Index[chemotaxis$Strain=="Glt-4"]
Glt5_octanol_subset <- chemotaxis$Octanol.Index[chemotaxis$Strain=="Glt-5"]

# Averages and standard deviation for chemotaxis - NaCl
wildtype_nacl_subset <- chemotaxis$NaCl.Index[chemotaxis$Strain=="wildtype"]
Glt3.Glt1_nacl_subset <- chemotaxis$NaCl.Index[chemotaxis$Strain=="Glt-3; Glt-1"]
Glt4_nacl_subset <- chemotaxis$NaCl.Index[chemotaxis$Strain=="Glt-4"]
Glt5_nacl_subset <- chemotaxis$NaCl.Index[chemotaxis$Strain=="Glt-5"]

# Plots chemotaxis - NaCl and octanol
par(las=1)
plot(seq(0.8, 1.2, length.out=12), wildtype_nacl_subset, main="", col=colors[1], pch=16, xlim=c(0.5,9.5), ylim=c(min(chemotaxis$Octanol.Index)-0.1, max(chemotaxis$NaCl.Index)+0.1), xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=c(1:4, 6:9), labels=rep("",8))
text(x = seq(1-0.5, 9-0.5, by=1), par("usr")[3] - 0.2, labels = c("Wildtype", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5", "" , "Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
axis(3, at=c(2.5,7.5), labels=c("NaCl", "Octanol"))
abline(h=0)
points(seq(1.8, 2.2, length.out=12), Glt3.Glt1_nacl_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=12), Glt4_nacl_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=12), Glt5_nacl_subset, col=colors[4], pch=16)
points(c(0.6, 1.4), rep(mean(wildtype_nacl_subset),2), type="l", lwd=2, col=colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_nacl_subset),2), type="l", lwd=2, col=colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_nacl_subset),2), type="l", lwd=2, col=colors[3])
points(c(3.6, 4.4), rep(mean(Glt5_nacl_subset),2), type="l", lwd=2, col=colors[4])
points(seq(5.8, 6.2, length.out=12), wildtype_octanol_subset, col=colors[1], pch=16)
points(seq(6.8, 7.2, length.out=12), Glt3.Glt1_octanol_subset, col=colors[2], pch=16)
points(seq(7.8, 8.2, length.out=12), Glt4_octanol_subset, col=colors[3], pch=16)
points(seq(8.8, 9.2, length.out=12), Glt5_octanol_subset, col=colors[4], pch=16)
points(c(5.6, 6.4), rep(mean(wildtype_octanol_subset),2), type="l", lwd=2, col=colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_octanol_subset),2), type="l", lwd=2, col=colors[2])
# Plots ethanol chemotaxis
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_ethanol_subset, main="", col=colors[1], pch=16, xlim=c(0.5,4.5), ylim=c(0,max(ethanol_chemo$Chemotactic.Index)+0.1), xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=1:4, labels=rep("",4))
text(x = seq(1, 4, by=1), par("usr")[3] - 0.03, labels = c("Wildtype", "+* Glt-1; Glt-3", ++* Glt-4", ++* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
points(seq(1.8, 2.2, length.out=6), Glt3.Glt1_ethanol_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=6), Glt4_ethanol_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=6), Glt5_ethanol_subset, col=colors[4], pch=16)
points(c(0.7, 1.3), rep(mean(wildtype_ethanol_subset),2), type="l", lwd=2,
col=colors[1])
points(c(1.7, 2.3), rep(mean(Glt3.Glt1_ethanol_subset),2), type="l", lwd=2,
col=colors[2])
points(c(2.7, 3.3), rep(mean(Glt4_ethanol_subset),2), type="l", lwd=2,
col=colors[3])
points(c(3.7, 4.3), rep(mean(Glt5_ethanol_subset),2), type="l", lwd=2,
col=colors[4])

# # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # #
# Read in chemotaxis csv file
butanone_assoc <- read.csv(file="CSV Files/Butanone Food Associative Thesis.csv",
header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_butanone_pre <- lm(Pre.Chemotactic.Index~Strain, data=butanone_assoc)
lm_butanone_post <- lm(Post.Chemotactic.Index~Strain, data=butanone_assoc)

# ANOVA
anova_butanone_pre <- aov(lm_butanone_pre)
anova_butanone_post <- aov(lm_butanone_post)

summary(anova_butanone_pre)
summary(anova_butanone_post)

# Post-Hoc Tukey Test
TukeyHSD(anova_butanone_pre)
TukeyHSD(anova_butanone_post)

# Averages and standard deviation for butanone pre
wildtype_butanone_pre_subset <- butanone_assoc$Pre.Chemotactic.Index[butanone_assoc$Strain=="wildtype"]
Glt3.Glt1_butanone_pre_subset <- butanone_assoc$Pre.Chemotactic.Index[butanone_assoc$Strain=="glt-3; glt-1"]
Glt4_butanone_pre_subset <- butanone_assoc$Pre.Chemotactic.Index[butanone_assoc$Strain=="glt-4"]
Glt5_butanone_pre_subset <- butanone_assoc$Pre.Chemotactic.Index[butanone_assoc$Strain=="glt-5"]

# Averages and standard deviation for butanone post
wildtype_butanone_post_subset <- butanone_assoc$Post.Chemotactic.Index[butanone_assoc$Strain=="wildtype"]
Glt3.Glt1_butanone_post_subset <- butanone_assoc$Post.Chemotactic.Index[butanone_assoc$Strain=="glt-3; glt-1"]
Glt4_butanone_post_subset <- butanone_assoc$Post.Chemotactic.Index[butanone_assoc$Strain=="glt-4"]
Glt5_butanone_post_subset <- butanone_assoc$Post.Chemotactic.Index[butanone_assoc$Strain=="glt-5"]
Post.Chemotactic.Index[butanone_assoc$Strain=="glt-3; glt-1"]
Glt4_butanone_post_subset <- butanone_assoc$
Post.Chemotactic.Index[butanone_assoc$Strain=="glt-4"]
Glt5_butanone_post_subset <- butanone_assoc$
Post.Chemotactic.Index[butanone_assoc$Strain=="glt-5"]

# Plot butanone chemotaxis
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_butanone_pre_subset, 
main="", col=colors[1], pch=16, xlim=c(0.5,5.5), 
ylim=c(min(butanone_assoc$Pre.Chemotactic.Index)-0.1, max(butanone_assoc$Post.Chemotactic.Index)+0.1), 
xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=c(1:4, 6:9), labels=rep("",8))
text(x = seq(1-0.5, 9-0.5, by=1), par("usr")[3] - 0.13, 
labels = c("Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5", " ", "Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
axis(3, at=c(2.5,7.5), labels=c("Pre", "Post"))
abline(h=0)

points(seq(1.8, 2.2, length.out=10), Glt3.Glt1_butanone_pre_subset, 
col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=10), Glt4_butanone_pre_subset, 
col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=10), Glt5_butanone_pre_subset, 
col=colors[4], pch=16)

points(c(0.6, 1.4), rep(mean(wildtype_butanone_pre_subset),2), 
type="l", lwd=2, col=colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_butanone_pre_subset),2), 
type="l", lwd=2, col=colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_butanone_pre_subset),2), 
type="l", lwd=2, col=colors[3])
points(c(3.6, 4.4), rep(mean(Glt5_butanone_pre_subset),2), 
type="l", lwd=2, col=colors[4])

points(seq(5.8, 6.2, length.out=10), wildtype_butanone_post_subset, 
col=colors[1], pch=16)
points(seq(6.8, 7.2, length.out=10), Glt3.Glt1_butanone_post_subset, 
col=colors[2], pch=16)
points(seq(7.8, 8.2, length.out=10), Glt4_butanone_post_subset, 
col=colors[3], pch=16)
points(seq(8.8, 9.2, length.out=10), Glt5_butanone_post_subset, 
col=colors[4], pch=16)
points(c(5.6, 6.4), rep(mean(wildtype_butanone_post_subset),2),
  type="l", lwd=2, col=colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_butanone_post_subset),2),
  type="l", lwd=2, col=colors[2])
points(c(7.6, 8.4), rep(mean(Glt4_butanone_post_subset),2),
  type="l", lwd=2, col=colors[3])
points(c(8.6, 9.4), rep(mean(Glt5_butanone_post_subset),2),
  type="l", lwd=2, col=colors[4])

# # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # #
# Read in chemotaxis csv file
ethanol_assoc <- read.csv(file="CSV Files/Ethanol Associative Thesis.csv",
  header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_ethanol_a <- lm(Chemotactic.Index~Strain, data=ethanol_assoc)

# ANOVA
anova_ethanol_a <- aov(lm_ethanol_a)
summary(anova_ethanol_a)

# Post-Hoc Tukey Test
TukeyHSD(anova_ethanol_a)

# Averages and standard deviation for ethanol_assoc
wildtype_ethanol_a_subset <- ethanol_assoc$Chemotactic.Index[ethanol_assoc$Strain=="wildtype"]
Glt3.Glt1_ethanol_a_subset <- ethanol_assoc$Chemotactic.Index[ethanol_assoc$Strain=="glt-3; glt-1"]
Glt4_ethanol_a_subset <- ethanol_assoc$Chemotactic.Index[ethanol_assoc$Strain=="glt-4"]
Glt5_ethanol_a_subset <- ethanol_assoc$Chemotactic.Index[ethanol_assoc$Strain=="glt-5"]

# Plots ethanol associative learning
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_butanone_pre_subset,
  main="", col=colors[1], pch=16, xlim=c(0.5,9.5),
  xlab="Strain", ylab="Chemotactic Index"")
points(c(5.6, 6.4), rep(mean(wildtype_butanone_post_subset),2),
  type="l", lwd=2, col=colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_butanone_post_subset),2),
  type="l", lwd=2, col=colors[2])
points(c(7.6, 8.4), rep(mean(Glt4_butanone_post_subset),2),
  type="l", lwd=2, col=colors[3])
points(c(8.6, 9.4), rep(mean(Glt5_butanone_post_subset),2),
  type="l", lwd=2, col=colors[4])
ylim = c(min(butanone_assoc$Pre.Chemotactic.Index) - 0.1, max(ethanol_assoc$Chemotactic.Index) + 0.1), xaxt = "n", xlab = "", ylab = "Chemotactic Index")
axis(1, at = c(1:4, 6:9), labels = rep("", 8))
text(x = seq(1-0.5, 9-0.5, by = 1), par("usr")[3] - 0.12, labels = c("Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5", "", "Wildtype", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
axis(3, at = c(2.5, 7.5), labels = c("Pre", "Post"))
abline(h = 0)
points(seq(1.8, 2.2, length.out = 10), Glt3.Glt1_butanone_pre_subset, col = colors[2], pch = 16)
points(seq(2.8, 3.2, length.out = 10), Glt4_butanone_pre_subset, col = colors[3], pch = 16)
points(seq(3.8, 4.2, length.out = 10), Glt5_butanone_pre_subset, col = colors[4], pch = 16)
points(c(0.6, 1.4), rep(mean(wildtype_butanone_pre_subset), 2), type = "l", lwd = 2, col = colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_butanone_pre_subset), 2), type = "l", lwd = 2, col = colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_butanone_pre_subset), 2), type = "l", lwd = 2, col = colors[3])
points(c(3.6, 4.4), rep(mean(Glt5_butanone_pre_subset), 2), type = "l", lwd = 2, col = colors[4])
points(seq(5.8, 6.2, length.out = 10), wildtype_ethanol_a_subset, col = colors[1], pch = 16)
points(seq(6.8, 7.2, length.out = 6), Glt3.Glt1_ethanol_a_subset, col = colors[2], pch = 16)
points(seq(7.8, 8.2, length.out = 6), Glt4_ethanol_a_subset, col = colors[3], pch = 16)
points(seq(8.8, 9.2, length.out = 6), Glt5_ethanol_a_subset, col = colors[4], pch = 16)
points(c(5.6, 6.4), rep(mean(wildtype_ethanol_a_subset), 2), type = "l", lwd = 2, col = colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_ethanol_a_subset), 2), type = "l", lwd = 2, col = colors[2])
points(c(7.6, 8.4), rep(mean(Glt4_ethanol_a_subset), 2), type = "l", lwd = 2, col = colors[3])
points(c(8.6, 9.4), rep(mean(Glt5_ethanol_a_subset), 2), type = "l", lwd = 2, col = colors[4])
# Read in chemotaxis csv file
nicotine_chemo <- read.csv(file="CSV Files/Nicotine Chemotaxis Thesis.csv", header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_nicotine <- lm(Chemotactic.Index~Strain, data=nicotine_chemo)

# ANOVA
anova_nicotine <- aov(lm_nicotine)

summary(anova_nicotine)

# Post-Hoc Tukey Test
TukeyHSD(anova_nicotine)

# Averages and standard deviation for nicotine_chemo
wildtype_nicotine_subset <- nicotine_chemo$Chemotactic.Index[nicotine_chemo$Strain=="wildtype"]
Glt3.Glt1_nicotine_subset <- nicotine_chemo$Chemotactic.Index[nicotine_chemo$Strain=="glt-3; glt-1"]
Glt4_nicotine_subset <- nicotine_chemo$Chemotactic.Index[nicotine_chemo$Strain=="glt-4"]
Glt5_nicotine_subset <- nicotine_chemo$Chemotactic.Index[nicotine_chemo$Strain=="glt-5"]

# Plots nicotine chemotaxis
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_nicotine_subset, main="", col=colors[1], pch=16, xlab="", ylab="Chemotactic Index")
points(seq(1.8, 2.2, length.out=6), Glt3.Glt1_nicotine_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=6), Glt4_nicotine_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=6), Glt5_nicotine_subset, col=colors[4], pch=16)
points(c(0.7, 1.3), rep(mean(wildtype_nicotine_subset),2), type="l", lwd=2, col=colors[1])
# Read in chemotaxis csv file
nicotine_assoc <- read.csv(file="CSV Files/Nicotine Associative Thesis.csv", header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_nicotine_a <- lm(Chemotactic.Index~Strain, data=nicotine_assoc)

# ANOVA
anova_nicotine_a <- aov(lm_nicotine_a)
summary(anova_nicotine_a)

# Post-Hoc Tukey Test
TukeyHSD(anova_nicotine_a)

# Averages and standard deviation for nicotine_assoc
wildtype_nicotine_a_subset <- nicotine_assoc$Chemotactic.Index[nicotine_assoc$Strain=="wildtype"]
Glt3.Glt1_nicotine_a_subset <- nicotine_assoc$Chemotactic.Index[nicotine_assoc$Strain=="glt-3; glt-1"]
Glt4_nicotine_a_subset <- nicotine_assoc$Chemotactic.Index[nicotine_assoc$Strain=="glt-4"]
Glt5_nicotine_a_subset <- nicotine_assoc$Chemotactic.Index[nicotine_assoc$Strain=="glt-5"]

# Plots nicotine associative learning
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_nicotine_a_subset, main="", col=colors[1], pch=16, xlim=c(0.5,9.5), ylim=c(min(butanone_assoc$Pre.Chemotactic.Index)-0.1, max(nicotine_assoc$Chemotactic.Index)+0.1), xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=c(1:4, 6:9), labels=rep("",8))
text(x = seq(1-0.5, 9-0.5, by=1), par("usr")[3] - 0.12, labels = c("Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5", "", "Wildtype", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
```r
axis(3, at=c(2.5,7.5), labels=c("Pre", "Post"))
abline(h=0)

points(seq(1.8, 2.2, length.out=10), Glt3.Glt1_butanone_pre_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=10), Glt4_butanone_pre_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=10), Glt5_butanone_pre_subset, col=colors[4], pch=16)

points(c(0.6, 1.4), rep(mean(wildtype_butanone_pre_subset),2), type="l", lwd=2, col=colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_butanone_pre_subset),2), type="l", lwd=2, col=colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_butanone_pre_subset),2), type="l", lwd=2, col=colors[3])
points(c(3.6, 4.4), rep(mean(Glt5_butanone_pre_subset),2), type="l", lwd=2, col=colors[4])

points(seq(5.8, 6.2, length.out=10), wildtype_nicotine_a_subset, col=colors[1], pch=16)
points(seq(6.8, 7.2, length.out=6), Glt3.Glt1_nicotine_a_subset, col=colors[2], pch=16)
points(seq(7.8, 8.2, length.out=6), Glt4_nicotine_a_subset, col=colors[3], pch=16)
points(seq(8.8, 9.2, length.out=6), Glt5_nicotine_a_subset, col=colors[4], pch=16)

points(c(5.6, 6.4), rep(mean(wildtype_nicotine_a_subset),2), type="l", lwd=2, col=colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_nicotine_a_subset),2), type="l", lwd=2, col=colors[2])
points(c(7.6, 8.4), rep(mean(Glt4_nicotine_a_subset),2), type="l", lwd=2, col=colors[3])
points(c(8.6, 9.4), rep(mean(Glt5_nicotine_a_subset),2), type="l", lwd=2, col=colors[4])

# # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # #

# Read in chemotaxis csv file
butanone_control <- read.csv(file="CSV Files/Butanone Control Thesis.csv", header=TRUE, stringsAsFactors=FALSE)

# Performs a least squares regression
lm_butanone_control <- lm(Chemotactic.Index~Strain, data=buchanone_control)
```
# ANOVA
anova_butanone_control <- aov(lm_butanone_control)
summary(anova_butanone_control)

# Post-Hoc Tukey Test
TukeyHSD(anova_butanone_control)

# Averages and standard deviation for butanone_control
wildtype_butanone_control_subset <- butanone_control$Chemotactic.Index[butanone_control$Strain=="wildtype"]
Glt3.Glt1_butanone_control_subset <- butanone_control$Chemotactic.Index[butanone_control$Strain=="glt-3; glt-1"]
Glt4_butanone_control_subset <- butanone_control$Chemotactic.Index[butanone_control$Strain=="glt-4"]
Glt5_butanone_control_subset <- butanone_control$Chemotactic.Index[butanone_control$Strain=="glt-5"]

# Plots butanone control
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_butanone_pre_subset, main="", col=colors[1], pch=16, xlim=c(0.5,9.5), ylim=c(min(butanone_assoc$Pre.Chemotactic.Index)-0.1,0), xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=c(1:4, 6:9), labels=rep("",8))
text(x = seq(1-0.5, 9-0.5, by=1), par("usr")[3] - 0.07, labels = c("Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5", "", "Wildtype", "* Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)
axis(3, at=c(2.5,7.5), labels=c("Pre", "Post"))
points(seq(1.8, 2.2, length.out=10), Glt3.Glt1_butanone_pre_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=10), Glt4_butanone_pre_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=10), Glt5_butanone_pre_subset, col=colors[4], pch=16)
points(c(0.6, 1.4), rep(mean(wildtype_butanone_pre_subset),2), type="l", lwd=2, col=colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_butanone_pre_subset),2), type="l", lwd=2, col=colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_butanone_pre_subset),2), type="l", lwd=2, col=colors[3])
points(c(3.6, 4.4), rep(mean(Glt5_butanone_pre_subset),2), type="l", lwd=2, col=colors[4])

59
points(seq(5.8, 6.2, length.out=5), wildtype_butanone_control_subset, col=colors[1], pch=16)
points(seq(6.8, 7.2, length.out=5), Glt3.Glt1_butanone_control_subset, col=colors[2], pch=16)
points(seq(7.8, 8.2, length.out=5), Glt4_butanone_control_subset, col=colors[3], pch=16)
points(seq(8.8, 9.2, length.out=5), Glt5_butanone_control_subset, col=colors[4], pch=16)
points(c(5.6, 6.4), rep(mean(wildtype_butanone_control_subset),2), type="l", lwd=2, col=colors[1])
points(c(6.6, 7.4), rep(mean(Glt3.Glt1_butanone_control_subset),2), type="l", lwd=2, col=colors[2])
points(c(7.6, 8.4), rep(mean(Glt4_butanone_control_subset),2), type="l", lwd=2, col=colors[3])
points(c(8.6, 9.4), rep(mean(Glt5_butanone_control_subset),2), type="l", lwd=2, col=colors[4])

# # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # #
# Plots pre-association for butanone
# # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # # #
par(las=1)
plot(seq(0.8, 1.2, length.out=10), wildtype_butanone_pre_subset, main="", col=colors[1], pch=16, xlim=c(0.5,4.5), ylim=c(min(butanone_assoc$Pre.Chemotactic.Index)-0.1,0), xaxt="n", xlab="", ylab="Chemotactic Index")
axis(1, at=1:4, labels=rep("",4))
text(x = seq(1, 4, by=1), par("usr")[3] - 0.07, labels = c("Wildtype", "Glt-1; Glt-3", "* Glt-4", "* Glt-5"), srt = 45, pos = 1, xpd = TRUE)

points(seq(1.8, 2.2, length.out=10), Glt3.Glt1_butanone_pre_subset, col=colors[2], pch=16)
points(seq(2.8, 3.2, length.out=10), Glt4_butanone_pre_subset, col=colors[3], pch=16)
points(seq(3.8, 4.2, length.out=10), Glt5_butanone_pre_subset, col=colors[4], pch=16)
points(c(0.6, 1.4), rep(mean(wildtype_butanone_pre_subset),2), type="l", lwd=2, col=colors[1])
points(c(1.6, 2.4), rep(mean(Glt3.Glt1_butanone_pre_subset),2), type="l", lwd=2, col=colors[2])
points(c(2.6, 3.4), rep(mean(Glt4_butanone_pre_subset),2), type="l", lwd=2, col=colors[3])
8.2 Data Visualization and Analysis for Non-Associative Learning (R Code)

```r
# Velocity of worms
combine_csvs <- function(ID, frame, x, y) {
  # Removes any rows with missing IDs, frames, x-values, and y-values
  ID <- ID[is.na(ID) == F]
  frame <- frame[is.na(frame) == F]
  x <- x[is.na(x) == F]
  y <- y[is.na(y) == F]
  # Combines data into one dataframe
  data <- data.frame(ID, frame, x, y)
  return(data)
}

get_velocities <- function(dataframe) {
  # Calculates time between frames
  time_between_frames <- 0.033
  # Obtains the number of worms in the file
  num_worms <- length(unique(dataframe$ID))
  # Creates placeholder vectors for the time and distance
  all_times <- rep(NA, num_worms)
  all_distances <- rep(NA, num_worms)
  # Calculates how many frames are in the file
  most_frames <- max(dataframe$frame)

  for (ii in 1:length(unique(dataframe$ID))) {
    # Obtains all the unique IDs
    this_ID <- unique(dataframe$ID)[ii]

    # Loops through worm by ID
    current_worm <- dataframe[dataframe$ID == this_ID,]
    current_distance <- rep(NA, (nrow(current_worm) - 1))

    for (jj in 1:nrow(current_worm) - 1) {
      # Calculates the distance traveled using the distance formula
      temp <- sqrt((current_worm$x[jj + 1] - current_worm$x[jj])^2 +
                    (current_worm$y[jj + 1] - current_worm$y[jj])^2)
      current_distance[jj] <- temp
    }
  }
}
```

```
# Adds all distances to get total distance
current_distance <- sum(current_distance)
# Adds all times to get total time
all_times[ii] <- (nrow(current_worm)-1)*time_between_frames
# Inserts total distance into vector of all distances
all_distances[ii] <- current_distance
}

# Reassigns ID number
ID_num <- seq(0, num_worms-1, by=1)
# Outputs dataframe of ID number, total distance, and total time
output <- data.frame(ID_num, all_distances, all_times)
# Calculates velocity
output$all_velocities <- output$all_distances / output$all_times
return(output)

# Reads in the wildtype files
wt_ID <- read.csv("WT-id.csv")
wt_frame <- read.csv("WT-timestamp_frame.csv")
wtx <- read.csv("WT-ximage.csv")
wt_y <- read.csv("WT-yimage.csv")

# Combines the wildtype files
wt_data <- combine_csvs(wt_ID, wt_frame, wt_x, wt_y)

# Separates the file into the 5 seconds after each tap
wt_tap_1 <- wt_data[wt_data$frame %in% 5:145, ]
wttap_2 <- wt_data[wt_data$frame %in% 155:295, ]
wttap_3 <- wt_data[wt_data$frame %in% 305:445, ]
wttap_4 <- wt_data[wt_data$frame %in% 455:595, ]
wttap_5 <- wt_data[wt_data$frame %in% 605:745, ]
wttap_6 <- wt_data[wt_data$frame %in% 755:895, ]
wttap_7 <- wt_data[wt_data$frame %in% 905:1045, ]
wttap_8 <- wt_data[wt_data$frame %in% 1055:1195, ]
wttap_9 <- wt_data[wt_data$frame %in% 1205:1345, ]
wttap_10 <- wt_data[wt_data$frame %in% 1355:1495, ]

# Combines all the wildtype taps into a list
wt_all_taps <- list(wt_tap_1, wt_tap_2, wt_tap_3, wt_tap_4, wt_tap_5, wt_tap_6, wt_tap_7, wt_tap_8, wt_tap_9, wt_tap_10)
# Makes a placeholder list
wt_temp <- list()

# Calculates velocities at each tap
for(ii in 1:length(wt_all_taps)) {
  wt_temp[[ii]] <- get_velocities(wt_all_taps[[ii]])$all_velocities
}

# Removes any velocities that are 0 (dead worm) or NA (error)
for(ii in 1:length(wt_all_taps)) {
  wt_temp[[ii]] <- wt_temp[[ii]][wt_temp[[ii]] != 0]
  wt_temp[[ii]] <- wt_temp[[ii]][is.na(wt_temp[[ii]]) == FALSE]
}

# Reads in the GLT-3 files
glt3_ID <- read.csv("GLT-3-id.csv")
glt3_frame <- read.csv("GLT-3-timestamp_frame.csv")
glt3_x <- read.csv("GLT-3-ximage.csv")
glt3_y <- read.csv("GLT-3-yimage.csv")

# Combines the GLT-3 files
glt3_data <- combine_csvs(glt3_ID, glt3_frame, glt3_x, glt3_y)

# Separates the file into the 5 seconds after each tap
glt3_tap_1 <- glt3_data[glt3_data$frame %in% 5:145,]
glt3_tap_2 <- glt3_data[glt3_data$frame %in% 155:295,]
glt3_tap_3 <- glt3_data[glt3_data$frame %in% 305:445,]
glt3_tap_4 <- glt3_data[glt3_data$frame %in% 455:595,]
glt3_tap_5 <- glt3_data[glt3_data$frame %in% 605:745,]
glt3_tap_6 <- glt3_data[glt3_data$frame %in% 755:895,]
glt3_tap_7 <- glt3_data[glt3_data$frame %in% 905:1045,]
glt3_tap_8 <- glt3_data[glt3_data$frame %in% 1055:1195,]
glt3_tap_9 <- glt3_data[glt3_data$frame %in% 1205:1345,]
glt3_tap_10 <- glt3_data[glt3_data$frame %in% 1355:1495,]

# Combines all GLT-3 taps into a list
glt3_all_taps <- list(glt3_tap_1, glt3_tap_2, glt3_tap_3, glt3_tap_4, glt3_tap_5,
glt3_tap_6, glt3_tap_7, glt3_tap_8, glt3_tap_9, glt3_tap_10)

# Creates placeholder list
glt3_temp <- list()

# Calculates the velocities at each tap
for(ii in 1:length(glt3_all_taps)) {
  glt3_temp[[ii]] <- get_velocities(glt3_all_taps[[ii]])$all_velocities
# Removes any velocities that are 0 (dead worm)
for(ii in 1:length(glt3_all_taps)) {
    glt3_temp[[ii]] <- glt3_temp[[ii]][glt3_temp[[ii]] != 0]
}

# Reads in the GLT-5 files
for(ii in 1:length(glt5_all_taps)) {
    glt5_temp[[ii]] <- get_velocities(glt5_all_taps[[ii]])$all_velocities
    glt5_temp[[ii]] <- glt5_temp[[ii]][glt5_temp[[ii]] != 0]
}
# Creates vector of velocities and strain for first tap
tap1_vel <- c(wt_temp[[1]], glt3_temp[[1]], glt5_temp[[1]])
tap1_strain <- c(rep("wt", length(wt_temp[[1]])), rep("glt3",
length(glt3_temp[[1]])), rep("glt5", length(glt5_temp[[1]]))))

# Runs a linear regression followed by Tukey for first tap
lm_tap1 <- lm(tap1_vel~tap1_strain)
anova_tap1 <- aov(lm_tap1)
TukeyHSD(anova_tap1)

# Creates vector of velocities and strain for fourth tap
tap4_vel <- c(wt_temp[[4]], glt3_temp[[4]], glt5_temp[[4]])
tap4_strain <- c(rep("wt", length(wt_temp[[4]])), rep("glt3",
length(glt3_temp[[4]])), rep("glt5", length(glt5_temp[[4]]))))

# Runs a linear regression followed by Tukey for fourth tap
lm_tap4 <- lm(tap4_vel~tap4_strain)
anova_tap4 <- aov(lm_tap4)
TukeyHSD(anova_tap4)

# Creates vector of velocities and strain for seventh tap
tap7_vel <- c(wt_temp[[7]], glt3_temp[[7]], glt5_temp[[7]])
tap7_strain <- c(rep("wt", length(wt_temp[[7]])), rep("glt3",
length(glt3_temp[[7]])), rep("glt5", length(glt5_temp[[7]]))))

# Runs a linear regression followed by Tukey for seventh tap
lm_tap7 <- lm(tap7_vel~tap7_strain)
anova_tap7 <- aov(lm_tap7)
TukeyHSD(anova_tap7)

# Creates vector of velocities and strain for tenth tap
tap10_vel <- c(wt_temp[[10]], glt3_temp[[10]], glt5_temp[[10]])
tap10_strain <- c(rep("wt", length(wt_temp[[10]])), rep("glt3",
length(glt3_temp[[10]])), rep("glt5", length(glt5_temp[[10]]))))

# Runs a linear regression followed by Tukey for tenth tap
lm_tap10 <- lm(tap10_vel~tap10_strain)
anova_tap10 <- aov(lm_tap10)
TukeyHSD(anova_tap10)
# This is the average velocity of wildtype worms at the tenth (last) tap
standardization_value <- mean(wt_temp[[10]])

# Standardizes velocities relative to the last tap for wildtype
wt_standardized <- list()
for(ii in 1:length(wt_temp)) {
    wt_standardized[[ii]] <- wt_temp[ii]/standardization_value
}

# Standardizes velocities relative to the last tap for wildtype
mlt3_standardized <- list()
for(ii in 1:length(mlt3_temp)) {
    mlt3_standardized[[ii]] <- mlt3_temp[ii]/standardization_value
}

# Standardizes velocities relative to the last tap for wildtype
mlt5_standardized <- list()
for(ii in 1:length(mlt5_temp)) {
    mlt5_standardized[[ii]] <- mlt5_temp[ii]/standardization_value
}

# Calculates the mean standardized velocity for each tap
wt_means <- rep(NA, length(wt_standardized))
for(ii in 1:length(wt_standardized)) {
    wt_means[ii] <- mean(wt_standardized[[ii]])
}

# Calculates the mean standardized velocity for each tap
mlt3_means <- rep(NA, length(mlt3_standardized))
for(ii in 1:length(mlt3_standardized)) {
    mlt3_means[ii] <- mean(mlt3_standardized[[ii]])
}

# Calculates the mean standardized velocity for each tap
mlt5_means <- rep(NA, length(mlt5_standardized))
for(ii in 1:length(mlt5_standardized)) {
    mlt5_means[ii] <- mean(mlt5_standardized[[ii]])
}

velocity <- list()
strain <- list()
for (ii in 1:length(wt_standardized)) {
  velocity[ii] <- c(wt_standardized[ii], glt3_standardized[ii],
         glt5_standardized[ii])
  strain[ii] <- c(rep("wt", length(wt_standardized[ii])), rep("glt3",
         length(glt3_standardized[ii])), rep("glt5", length(glt5_standardized[ii])))
}

tap_number <- list()
for (ii in 1:length(wt_standardized)) {
  tap_number[ii] <- c(rep(ii, length(wt_standardized[ii])),
         rep(ii, length(glt3_standardized[ii])), rep(ii, length(glt5_standardized[ii])))
}

# Unlists velocity, strain, and tap number so they are
# manipulate-able as vectors
velocity <- unlist(velocity)
strain <- unlist(strain)
tap_number <- unlist(tap_number)

# Creates a dataframe of velocity, tap number, and strain
tapping <- data.frame(velocity, tap_number, strain)

# Calculates the average velocity of each strain at each tap number
tapping.mean <- aggregate(tapping$velocity,
by = list(tapping$tap_number, tapping$strain), FUN = 'mean')

# Names the columns of the new dataframe
colnames(tapping.mean) <- c("tap_number","strain", "velocity")

# Performs an ANOVA and Tukey HSD on tap number within wildtype strain
tapping.wt.anova <- aov(velocity ~ as.factor(tap_number), data=tapping_wt_only)
TukeyHSD(tapping.wt.anova)

# Creates a subset of only the wildtype strain at taps 1, 4, 7, and 10
tapping_wt_only <- tapping[tapping$strain=="wt",]
tapping_wt_only_selected_taps <- tapping_wt_only[tapping_wt_only$tap_number==1 |
         tapping_wt_only$tap_number==4 |
         tapping_wt_only$tap_number==7 | tapping_wt_only$tap_number==10,]

# Creates a subset of only the GLT-3 strain at taps 1, 4, 7, and 10
tapping_glt3_only <- tapping[tapping$strain=="glt3",]
tapping_glt3_only_selected_taps <- tapping_glt3_only[tapping_glt3_only$tap_number==1 | tapping_glt3_only$tap_number==4 | tapping_glt3_only$tap_number==7 | tapping_glt3_only$tap_number==10,]

# Performs an ANOVA and Tukey HSD on tap number within GLT-3 strain
tapping.glt3.anova <- aov(velocity ~ as.factor(tap_number), data=tapping_glt3_only)
TukeyHSD(tapping.glt3.anova)

tapping_glt5_only <- tapping[tapping$strain=='glt5',]

tapping_glt5_only_selected_taps <- tapping_glt5_only[tapping_glt5_only$tap_number==1 | tapping_glt5_only$tap_number==4 | tapping_glt5_only$tap_number==7 | tapping_glt5_only$tap_number==10,]

# Performs an ANOVA and Tukey HSD on tap number within GLT-5 strain
tapping.glt5.anova <- aov(velocity ~ as.factor(tap_number), data=tapping_glt5_only)
TukeyHSD(tapping.glt5.anova)

# Plots relative velocity of wildtype strain at all taps and displays mean
par(las=1, mar=c(4,4,2,2)+0.1)
plot(1:10, col="white", ylim=c(0,65), xaxt="n", xlab="Tap Number", ylab="Relative Velocity")
axis(1, at=1:10, labels=1:10)
abline(h=1)
for (ii in 1:10) {
  points(seq(ii-0.2, ii+0.2, length.out=length(wt_standardized[[ii]])), wt_standardized[[ii]], pch=16, col="violetred4")
}
points(x=c(0.8, 0.8, 2.2, 2.2), y=c(59, 61, 61, 59), type="l", lwd=2)
text(x=1.5, y=63, labels="*", cex=2)
points(1:10, tapping.mean$velocity[tapping.mean$strain="wt"], type="l", col="violetred3", lwd=3)

# Plots relative velocity of GLT-3 strain at all taps and displays mean
par(las=1, mar=c(4,4,2,2)+0.1)
plot(1:10, col="white", ylim=c(0,30), xaxt="n", xlab="Tap Number",
ylab="Relative Velocity")
axis(1, at=1:10, labels=1:10)
abline(h=1)
for (ii in 1:10) {
    points(seq(ii-0.2, ii+0.2, length.out=length(glt3_standardized[[ii]])),
           glt3_standardized[[ii]], pch=16, col="dodgerblue4")
}
points(1:10, tapping.mean$velocity[tapping.mean$strain=="glt3"], t
       ype="l", col="dodgerblue3", lwd="3")
# Plots relative velocity of GLT-5 strain at all taps and displays mean
par(las=1, mar=c(4,4,2,2)+0.1)
plot(1:10, col="white", ylim=c(0,30), xaxt="n", xlab="Tap Number",
ylab="Relative Velocity")
axis(1, at=1:10, labels=1:10)
abline(h=1)
for (ii in 1:10) {
    points(seq(ii-0.2, ii+0.2, length.out=length(glt5_standardized[[ii]])),
           glt5_standardized[[ii]], pch=16, col="darkgoldenrod3")
}
points(1:10, tapping.mean$velocity[tapping.mean$strain=="glt5"], t
       ype="l", col="darkgoldenrod2", lwd="3")
# Plots mean relative velocity for all of the three strains at all 10 tapping timepoints
par(las=1, mar=c(4,4,2,2)+0.1)
plot(1:10, col="white", ylim=c(0,16), xaxt="n", xlab="Tap Number", ylab="Average Relative Velocity")
axis(1, at=1:10, labels=1:10)
abline(h=1)
points(1:10, tapping.mean$velocity[tapping.mean$strain=="wt"], t
       ype="l", col="violetred4", lwd="2")
points(1:10, tapping.mean$velocity[tapping.mean$strain=="glt3"], t
       ype="l", col="dodgerblue4", lwd="2")
points(1:10, tapping.mean$velocity[tapping.mean$strain=="glt5"], t
       ype="l", col="darkgoldenrod3", lwd="2")
text(x=1, y=tapping.mean$velocity[tapping.mean$strain=="glt3"][1], l
       abels="*", cex=2.5)
text(x=1, y=tapping.mean$velocity[tapping.mean$strain=="glt5"][1], l
       abels="*", cex=2.5)
text(x=10, y=tapping.mean$velocity[tapping.mean$strain=="glt3"][10], l
       abels="*", cex=2.5)
8.3 Arduino Code to Control Mechanical Tapper

8.3.1 Training Phase

// the setup function runs once when you press reset or power the board
void setup() {
  // initialize digital pin 13 as an output.
  pinMode(13, OUTPUT);
}

void loop() {
  for(int CounterOne = 0; CounterOne < 21; CounterOne++) {
    digitalWrite(13, HIGH); // turn the tapper on (HIGH is the voltage level)
    delay(100); // wait for 1/10 second
    digitalWrite(13, LOW); // turn the tapper off by making the voltage LOW
    delay(60000); // wait for a minute;
  }

delay(900000); // wait 15 minutes

  for(int CounterTwo = 0; CounterTwo < 20; CounterTwo++) {
    digitalWrite(13, HIGH);
    delay(100);
    digitalWrite(13, LOW);
    delay(60000);
  }

delay(900000);

  for(int CounterThree = 0; CounterThree < 4; CounterThree++) {
    digitalWrite(13, HIGH);
    delay(100);
    digitalWrite(13, LOW);
    delay(60000);
  }

delay(900000);

  for(int CounterFour = 0; CounterFour < 4; CounterFour++) {
    digitalWrite(13, HIGH);
    delay(100);
    digitalWrite(13, LOW);
    delay(60000);
  }

delay(900000);
8.3.2 Testing Phase

// the setup function runs once when you press reset or power the board
void setup() {
    // initialize digital pin 13 as an output.
    pinMode(13, OUTPUT);
}

void loop() {
    for(int CounterOne = 0; CounterOne < 11; CounterOne++) {
        digitalWrite(13, HIGH); // turn the tapper on (HIGH is the voltage level)
        delay(100); // wait for 1/10 second
        digitalWrite(13, LOW); // turn the tapper off by making the voltage LOW
        delay(30000); // wait for 30 seconds;
    }

    delay(900000); // wait 15 minutes
}