CHEMOSENSORY CONTEXT CONDITIONING IN

CAENORHABDITIS ELEGANS

by

Hsien Lee Lau

B.Sc., The University of British Columbia, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2010

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ABSTRACT

These studies were designed to investigate how environmental cues are associated during a non-associative learning process by studying chemosensory context conditioning for habituation in the nematode Caenorhabditis elegans. In chemosensory context conditioning for habituation animals that are trained and tested in the presence of either a taste or smell context cue show greater retention of habituation to tap stimuli when compared to animals trained and tested in different environments. This thesis is based on the work of Rankin (2000), in which taste (sodium acetate) context conditioning of habituation, extinction and latent inhibition of the cue were demonstrated. Here, I have shown context conditioning for an olfactory chemosensory cue (diacetyl) and dissociated the taste and smell pathways for this form of learning. odr-7 worms, with non-functional AWA olfactory chemosensory neuron (that detects diacetyl), showed short-term context conditioning to the taste but not to smell; the reverse was true for osm-3 worms with non-functional taste chemosensory neurons. This dissociation allows me to distinguish learning genes from genes involved in the detection of taste or smell. I also demonstrated long-term associative memory (24h) for context conditioning; context conditioning did not enhance normal long-term habituation, however, it produced memory in a training procedure that normally does not produce memory. My results showed that glr-1 (an AMPA-type ionotropic glutamate receptor subunit) and nmr-1 (an NMDA-type ionotropic glutamate receptor subunit) mutant worms did not show either short- or long-term context conditioning. To identify one site of plasticity, I showed that NMR-1 in the RIM interneurons was critical to produce short-term olfactory context conditioning. These studies lay the foundation to elucidate the cellular mechanisms of non-associative
and associative learning for both short- and long-term memory, and may provide insights into how interneurons integrate information from multiple sensory systems.
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<table>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxyl-methylisoxazole-4-propionate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
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<td>h</td>
<td>hours</td>
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<td>ISI</td>
<td>inter stimulus-interval</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>M</td>
<td>molarity</td>
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<td>min</td>
<td>minutes</td>
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<tr>
<td>ml</td>
<td>milliliters</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>s</td>
<td>seconds</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>VCR</td>
<td>video cassette recording</td>
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<td>VDS</td>
<td>visual danger stimulus</td>
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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Cathy, my supervisor, for positioning me here to write this thesis. Her care, academic counsel and personal advice have enriched my experience in her lab and for years to come. I liken her role in my life as my academic guardian, protecting me, nurturing me and giving me a swift kick in the butt as needed. Furthermore, her passion for research has been truly inspiring and has significantly diversified my interests and critical thinking skills. I would also like to thank my committee members comprised of Kurt Haas, Catherine Winstanley and Debbie Giaschi, all of whom offered great support and excellent input towards this thesis.

I have come to see my colleagues as members of my extended family, providing invaluable help, advice, support and friendship. One of them has played a pivotal role: Andrew Giles brought me into the light of science! Andrew Giles was my project supervisor during my undergraduate years and has remained a friend, mentor and role model whom I continue to seek advice and support from. I would like to remember and treasure the friendships I have fostered in this lab with Tiffany Timbers for her molecular biology support, her insightful questions and life changing views on food, Conny Lin for helping me with statistics and patiently teaching me molecular biology techniques, and Evan Ardiel for our many fruitful discussion about my experiments, science and EVERYthing else under the sun. I would also like to thank Jackie Law for holding down the lab tech fort, running the maze learning project and faithfully scoring my data, Nadia Ivanova, Serena Kooner, Aileen Wang, Rand Mahmoud, Jonathan Looi, MingMei Zhang, and Helena Yu for support in every way received in the lab.
Last but not least, I would like to thank my parents, Laurence Lau and Chui Han Wong Lau, and my brother, Keen Lau, for always doing what they think is best for me; without their guidance and support, I would not have the privileged life I am living today.
CHAPTER 1

1.1 General introduction

Traditionally learning theorists have distinguished between different forms of learning based on the training protocol used to produce learning. Based on training procedures, learning has been divided first into non-associative and associative learning. In a non-associative learning paradigm, the behavior changes in response to a single stimulus. The two main types of non-associative learning are habituation and sensitization. In habituation, extended or repeated exposure to a stimulus leads to response decrement; in sensitization, exposure to a stimulus leads to an increase in responding. In associative learning, an organism learns an association between two stimuli (Classical or Pavlovian Conditioning) or between a response and an outcome (Instrumental or Operant Conditioning). This review is about a form of classical conditioning, context conditioning.

The most familiar form of classical conditioning occurs when a neutral stimulus (conditioned stimulus, CS) is presented just before (and may overlap with) a second stimulus (unconditioned stimulus, US) that elicits an innate, often reflexive, response (unconditioned response, UR). Pavlov (1927) first studied this form of learning in dogs – a bell rang (CS) immediately before the dogs received food (US) that produced salivation (UR). After several pairings of the CS and US, the CS elicited salivation when presented without the US. Thus, when the dog heard the bell (CS) it salivated because it had associated the CS (bell) and US (food). This form of classical conditioning in which the CS is a discrete stimulus is called “cue conditioning”.

Context conditioning is a second form of classical conditioning in which aspects
of the training environment or chamber (e.g. smells, visual stimuli, floor textures, noises, etc…) that are present for the duration of the trial are associated with the presentation of a US which leads to a performance of a conditioned response when subsequently exposed to the same training environment. Context conditioning can occur on its own and has been studied in a number of different paradigms in organisms including rats (Walker et al., 2005b), pigs (de Jonge et al., 2008), humans (Marschner et al., 2008). Context conditioning can also occur with non-associative learning (habituation in crabs *Chasmagnathus*; Maldonado et al., 1997, and sensitization in *Aplysia*; Colwill et al., 1988b, a) or with associative learning (cue classical conditioning in mice; Maren, 2001, and in instrumental conditioning in snails; Haney and Lukowiak, 2001).

Although vertebrate studies in associative learning and memory have provided many theoretical models and practical applications, the overwhelming number and interconnectivity of neurons in vertebrate nervous systems makes it difficult to study at a neuronal level. To bridge this gap, the introduction of invertebrates as models for learning and memory has begun to bear fruit (Carew and Sahley, 1986). In invertebrates, the central nervous system is considered simple or basic compared to vertebrates; this is advantageous in research. In invertebrates, it is possible to identify accessible neurons underlying a behavior; this allows for the analysis of molecular changes in a neuron directly involved in the behavior of interest. Furthermore many neurotransmitters, proteins and their transduction pathways are largely conserved throughout phylogeny. Hence, the basic functions of memory are likely to be similar from invertebrates to mammals, and differences in mnemonic processes may simply be consequences of increased neuronal complexity. The subject of this thesis is context conditioning,
therefore in this Chapter I will review the research that has been published on context conditioning using invertebrate model systems.

1.2 Context conditioning in *Aplysia californica*

*Aplysia* is a marine mollusk (sea slug) that has been extensively used as a model system to study non-associative and associative learning (Pinsker et al., 1970; Hawkins et al., 2006). Although this chapter will focus primarily on context conditioning, a brief review of non-associative learning is needed.

Several behaviors have been widely used to study learning and memory in *Aplysia*, such as the defensive withdrawal (of gill, siphon, and mantle) reflex, tail withdrawal reflex, escape locomotion, feeding responses and head waving responses. Studies in context conditioning have focused on the defensive withdrawal reflexes, specifically the siphon withdrawal reflex. This reflex is induced by a mechanical stimulus to the siphon that causes the withdrawal of the siphon and mantle organs into the mantle cavity. Repeated stimulation of the siphon produces habituation, a decrement in the siphon and mantle organ withdrawal that can last from several minutes to days (Carew et al., 1972). When the animal is habituated to siphon stimulation, the delivery of a strong stimulus (e.g. electric shock) to the head or tail produces dishabituation of the defensive withdrawal reflex, an increase in siphon and mantle organ withdrawals (Pinsker et al., 1970). Sensitization, a form of non-associative learning, occurs when the sensitizing stimulus is presented to the slug that has not habituated to siphon stimulation. Sensitization is defined as a significant increase above baseline responding following a noxious, arousing stimulus. The neural circuit, synaptic, electrophysiological and molecular aspects of sensitization have been well studied.
These studies set the stage for understanding more complex forms of learning, such as context conditioning. To date, only two studies (Colwill et al., 1988b, a) have investigated context conditioning with sensitization in *Aplysia*. These studies used the siphon withdrawal reflex as a measure of context conditioning. During the training sessions, slugs were placed in two distinct contexts for 20 min each day for 8 days. One context was a smooth round bowl containing lemon-flavored seawater and the other was a rectangular chamber containing gently vibrating unscented seawater. Each day, the slugs received 4 moderate electric shocks (the US) in one of the contexts and no US in the other. Twenty-four hours after the training session, memory/context conditioning was tested by mechanically stimulating the siphon in one of the contexts; memory was assessed as the duration of siphon withdrawal. Slugs that were trained and tested in the same context showed significantly greater memory (greater duration of siphon withdrawal) compared to slugs that had received the shocks without the context training; the association between the context and electric shock could last for at least 48h (Colwill et al., 1988b).

Colwill et al. (1988a) demonstrated that slugs were being conditioned to the context and that the change in behavior was not due to other factors such as the sensitization of siphon withdrawal during training. If the context no longer predicted the US (extinction), no increase in siphon withdrawal would be observed when slugs were trained and tested in the same context. To extinguish the context-US association made during training, during the next two days, slugs were periodically placed in the context in which they had received the US during training, but were given no US, or they were left in their home tank. On the third day, when both groups were put into the context in which
they originally received the US only the group that remained in their home tank showed context conditioning, the group exposed to the context without shock did not. A second confirmation that control context was associated with the US was to show the learning phenomena of blocking. In blocking, if a CS (CS1) is already associated with a US, subsequent pairings of another CS (CS2) with the US will be impaired. In blocking, after slugs received the context (CS1) and electric shock (US) pairings, mechanical siphon stimulation (CS2) was paired with electric shock (US) 24h after the initial context-US training. Twenty-four hours later, they found that the formation of the CS2 (mechanical siphon stimulation)-US association was impaired only in the group that received the CS1 (context)–US association. Slugs that were trained and tested in different contexts showed the CS2-US association. These results show first, that Aplysia can demonstrate context conditioning and because it also shows extinction (no context conditioning when exposed to the CS without the US after training), this is a form of associative learning. Second, when slugs have been context conditioned, a context-US association is formed and this impairs (or “block”) subsequent associations of another CS2 to the US.

Many studies with mammals have shown that multiple pairings of CS’s and US’s can be associated with the context of those pairings (Balsam, 1985). Colwill et al. (1988a) showed that slugs could remember two different contexts in which two different types of CS-US pairings took place. Two groups of slugs received the same training in context A, in which mechanical siphon stimulation (CS) was always paired with electric shock (US). One of the groups of slugs then received only the mechanical siphon stimulation without any shocks (CS no US) in context B. The second group of slugs received both the CS and US in an unpaired fashion in context B. Twenty-four hours after
the training sessions, animals in both groups were tested with presentations of the CS in both context A and B. The group that was tested in the same context (A) in which it received the CS-US pairing showed significantly more memory retention (increased siphon withdrawal duration) compared to groups that were tested in the same context in which slugs received no US (group 1) or the unpaired CS-US procedure (group 2) (Colwill et al., 1988a). These experiments suggest that Aplysia have the ability to concurrently remember and associate the contexts of two different CS training procedures.

1.3 Context conditioning in *Hermisenda crassicornis*

Associative learning in the sea slug *Hermisenda* has been extensively studied using Classical Conditioning procedures (Crow, 2004). Identified neurons that are components in the CS and US pathways have been studied using biochemical, electrophysiological and pharmacological techniques in which surgical removal of these pathways allow for semi-intact analysis. These powerful tools have allowed researchers to study associative learning and investigate how neural circuits are modified by prior experience to produce changes in behavior. This has provided some unique insights into the mechanisms of context conditioning in this sea slug.

To study the role of context in learning and memory, *Hermisenda*’s innate response to high-speed rotation (US) was studied. When experiencing rotational forces, the slugs will contract their foot to initiate a clinging response. These responses are adaptive in their natural environment in order to feed and to survive stormy weather. Chemosensory context conditioning in *Hermisenda* was demonstrated by presenting 50 high-speed rotations (US) in either shrimp- or scallop-flavored water/context each day for
three days during training. Twenty-four hours later, on the fourth day, animals were tested by measuring the time it took for them to strike at shellfish meat (shrimp or scallop). The US rotations are aversive stimuli that normally increase the latency for approach and consumption of food. Slugs that were tested in the context (shrimp or scallop flavored water) that was paired with rotation (US) had a longer latency before striking food compared to those tested in context that was not paired with the US. Furthermore, in a preference test between the two contexts, conditioned *Hermissenda* preferred the context in which they did not receive the aversive US. Finally, slugs had a stronger defensive clinging response when they received a US in the context that was previously paired with the US compared to the context that was unpaired with the US (Rogers et al., 1996). The authors concluded that the slugs were making associations between the context and aversive US. One limitation of this study was that no control experiments were carried out to show that associative learning between the context and US was occurring. If associative learning was occurring between the context and US, an experiment demonstrating extinction should have been carried out by exposing the slugs to the US-reinforced context without US presentations for the 24 hours between training and testing. If associative learning was occurring, the slugs would learn that the context was not a good predictor of the US, hence, have decreased food-strike latency, no context preference, and decreased foot contractions in response to subsequent USs.

In a similar study to elucidate the cellular correlates and the possible sites of context conditioning in *Hermissenda*, Rogers and Matzel (1996) showed the learning phenomena of blocking in which the association of a CS (CS1) to a US can block subsequent associations of different CSs (CS2) to the same US. In this study, the
association between the context/CS1 (shrimp or scallop) and the aversive US (high-speed rotation) could block future associations between subsequent CS2 (light) with the same aversive US. This study also supported the associative nature of context conditioning by showing latent inhibition. In this learning phenomena, pre-exposure to the CS causes the animal to learn that the context (or CS) does not signal any biologically significant stimuli, hence, does not later associate the pre-exposed CS with other USs. Rogers and Matzel (1996) showed slugs do not show context conditioning to the CS when the CS is pre-exposed to the context before training (pairing of the context and aversive US).

Various sites of plasticity underlie context, light and US associations (McPhie et al., 1993). One site of chemosensory context conditioning plasticity is the type B photoreceptors since chemosensory pathways are known to interact synaptically to inhibit type B photoreceptors in the eye (Alkon et al., 1978). In semi-intact preparations of the neural circuit underlying chemosensory context conditioning, current-clamp recordings demonstrated that one of the primary storage sites of the light-rotation (context-US) association was in the medial B photoreceptor (Rogers and Matzel, 1996). Increases in excitability were observed after context-US training, this suggested that the B cell is a site of plasticity. However, both context-US paired and unpaired groups showed equivalent increases in B receptor excitability despite differential behavioral responding. Consistent with previous studies, the authors suggest that multiple sites of plasticity/integration must exist.

To further examine the cellular mechanisms of context conditioning in *Hermisenda*, Talk et al. (1999) used light instead of chemosensory cues as the context to study the cellular differences between cue and context conditioning. In cue conditioning,
the CS is discretely presented with the US, however in context conditioning, the CS is diffusely present throughout the presentation of the USs. Diffuse chemosensory cues in context conditioning are processed by a large network of unidentified neurons and cannot be presented in a discrete, on/off fashion as used in cue conditioning. Instead of using chemosensory cues, light can be presented in a highly controlled fashion and the network of neurons underlying vision is better understood than the neural network for chemosensation. Currently, it is not known why neural responses to temporally diffuse contextual signals throughout training are not extinguished during the intervals between US presentations. Conversely in cue (CS) conditioning, the presentation of the CS during the intervals between the US extinguishes CS-US association. In light-based context conditioning, *Hermisenda* were trained with three 15-minute sessions in which the slugs received 60 bursts of high-speed rotations (US) in 2 different contexts, dark or light. In a dark/light preference test, if the US were presented in the dark, slugs tended to prefer the light and vice versa if the US were presented in the light. To understand the neurophysiological differences between cue and context conditioning Talk et al. (1999) used *in vitro*, surgically excised preparations of the neural circuit underlying context and cue conditioning using current and calcium (calcium-indicating florescent dye Fura-2) recordings in the B photoreceptors. They found that after context and cue conditioning the neural circuit showed similar voltage/current and calcium changes (Talk et al., 1999). The authors concluded that a common mechanism might underlie cue and context conditioning. However, no behavioral data was correlated with the neurophysiological evidence presented for cue conditioning. As described earlier, changes in B receptor excitability did not correlate with the expression of context conditioning (Rogers et al.,
Context conditioning in *Hermissenda* is still debated. Jin et al. (2004) failed to see context conditioning in *Hermissenda* when they paired high-speed rotation (US) with shrimp- and scallop-flavored context. In a review, Farley et al. (2004) discussed the dangers of using food-related cues when studying context conditioning in *Hermissenda*. These dangers include altered feeding motivation and altered responses to prolonged food cues. In addition, non-associative factors such as sensory adaptation, habituation and sensitization were proposed to confound context conditioning experiments in *Hermissenda*.

1.4 Context conditioning in the honeybee *Apis Mellifera*

One behavioral paradigm that has been extensively used to study context conditioning in the honeybee is the proboscis extension response. In this learning paradigm, forger bees are caught and their antennae are presented with an unscented sucrose solution (US) that elicits a reflex response: the antennae move forward, mandibles open and the proboscis (tubular feeding/sucking organ) is extended. If a neutral odor (CS) is paired with the presentation of the sugar solution (US), bees form an association with between the odor and sugar solution. This results in an increase of proboscis extensions in the presence of the odor (CS) that was previously paired with the sucrose solution (Menzel, 1983).

Gerber and Menzel (2000) modified the proboscis extension response procedure to further explore the role of context in classical conditioning. They found that forward (context exposure for 55 seconds followed by 5-second US exposure) and not backward (US exposure for 5 seconds followed by 55-second context exposure) pairings of the
training chamber and sucrose (context-US) enhanced subsequent learning of the odor-sucrose (CS-US) association one day later. This was suggested to be an enhancement of CS-US memory consolidation. In this study, one would have predicted the learning phenomena of blocking to occur, in which prior training to one stimulus (context) will block conditioning to another stimulus (odor). However, Gerber and Smith (1998) did not observe blocking in a similar paradigm despite the predictions of current theories of conditioning. It was suggested that inter-modal stimulus (visual and odor) associations are not processed independently as seen in vertebrates, on the contrary, visual and olfactory stimuli interact in the honeybee. This hypothesis supports the finding that first associating the context to sucrose does not block, but facilitates subsequent odor-sucrose association. To further support the associative nature of context, partial extinction of context was observed: when honeybees remained in the training/testing chamber (context) between training and testing, a decrease in memory retention was observed when tested 3 min later; this was not the case if honeybees were taken out of the context between training and testing (Gerber et al., 1998).

The use of well-characterized behaviors, such as the proboscis extension response, can provide molecular insights into context conditioning in the honeybee. Pharmacological and protein inhibition (chilling) analyses have identified 3 areas of the bee brain as sites of convergence for olfactory and reward learning: the antennal lobes, mushroom bodies and lateral horn. Pharmacological manipulation of these sites can serve as a surrogate stimulus presentation. For example, injection of octopamine into any of the three sites can replace the sucrose reward in an olfactory conditioning experiment to later produce olfactory memories (Hammer and Menzel, 1998). Furthermore, these sites of
convergence have been used to demonstrate the role of protein synthesis, PKC and PKM signaling in short- middle- and long-term memory (Muller, 2000).

1.5 Context conditioning in *Lymnaea stagnalis*

*Lymnaea* are fresh water snails that are bi-modal breathers, capable of acquiring oxygen mainly through cutaneous respiration in normal oxygenated environments or through aerial respiration in hypoxic environments. *Lymnaea* has been used as a model for learning and memory since the necessity and sufficiency of a three-interneuron central pattern generator (CPG); this was demonstrated in the aerial respiration behaviors through cell-culture and *in vivo* transplantation procedures (Syed et al., 1992). As a result, conditioning procedures using the aerial respiration response have been developed and a large number of features of instrumental conditioning studied.

Negative reinforcement, a form of instrumental conditioning, is the increase of a behavior (keeping the pneumostome, a breathing pore, closed) in order to avoid an aversive stimulus (pneumostome stimulation). In order to reliably elicit the aerial respiration/opening of the pneumostome, snails have to be placed in water bubbled with nitrogen gas to create a hypoxic environment. In the hypoxic environment, the opened pneumostome is closed by applying a weak tactile stimulus, presented with a wooden stick, to the opened pneumostome. As a result of training, during the test session when the slugs are placed back into the hypoxic environment, there is decrease in pneumostome opening. Depending on the training procedure, the memory to keep their pneumostome closed during the hypoxic treatments can last for up to a month (Lukowiak et al., 1998).

Using the negative reinforcement procedure with either plain or carrot flavored
water as contexts, Haney and Lukowiak (2001) demonstrated that snails only showed memory for the pneumostome response if they were trained and 18h later tested in the same context. This was true for both plain and carrot flavored water. During each training session the pneumostome was poked each time the snail came up to the surface to breath. Long-term memory training consisted of three 15 min sessions 1h apart; this produced long-term memory 18h later. If snails were trained in the plain flavored beaker and tested 18h later in the carrot-flavored beaker, or vice versa, no memory was observed (Haney and Lukowiak, 2001).

In the training procedure above, context plays a critical role in long-term memory formation in *Lymnaea*. Parvez et al. (2006) extended these findings by using an intermediate-term training procedure to demonstrate memory reconsolidation in the snail. Memory reconsolidation is the reactivation of a previously consolidated memory during which the memory re-enters a labile stage requiring another round of consolidation or reconsolidation (Nader, 2003). To show memory reconsolidation, intermediate-term training was presented in the form of two 30-min training sessions 30 min apart. Twenty-four hours after the training session, a memory reactivation session was presented to the snail by putting them back in the training context and giving them one pneumostome poke when it opened. This reactivation session boosted the longevity of the memory from intermediate- to long-term memory. Memory was observed 24h after the reactivation session or 48h after the training session. This effect was only observed if the context was the same during both the training and memory reactivation session. If the training and reactivation sessions were carried out in different contexts (plain and carrot-flavored water or vice versa), no long-term memory was observed. Since reconsolidation is
sensitive to similar treatments that block consolidation (Nader et al., 2000; Duvarci et al., 2008), snails that were chilled to block protein synthesis immediately after intermediate-term training or immediately after the memory reactivation session did not show the boost from intermediate-term memory to long-term memory (Parvez et al., 2006). In a similar paradigm, Rosenegger et al. (2008) showed that pharmacologically increasing phosphorylation could produce long-term memory 24h after training in an intermediate-term memory procedure. This memory boost was achieved by either inhibiting protein phosphatase activity or by increasing protein kinase C (PKC) activity prior to intermediate-term training. This memory boost was not possible without the soma of the RPeD1 neuron, suggesting that a balance between phosphorylation and dephosphorylation in the RPeD1 neuron is critical for long-term memory formation (Rosenegger et al., 2008).

To further test the role of context (plain or carrot-flavored) in memory reconsolidation, snails were given long-term memory training followed by a 45 min reactivation training session three days later (Lukowiak et al., 2007). The long-term memory session consisted of three 45 minute sessions over a 1.5 day period; this training produced memory 5 days later (McComb et al., 2002). Immediately after the reactivation session the snails were presented with either the context they were trained in or a new context; this treatment resulted in memory being observed in both the new context and the context they were initially trained in. Lukowiak et al. (2007) suggest that the context in which memory reconsolidation occurs can alter the conditions under which the memory is expressed, resulting in memory infidelity. Whether the reconsolidation with the new context caused a generalization of the memory to any context is unclear.
However, blocking protein synthesis (by chilling) during the reconsolidation period (after the reactivation session) blocks the memory infidelity effect (Lukowiak et al., 2007).

### 1.6 Context conditioning In *Drosophila melanogaster*

Despite the large number of behaviors and the powerful genetics of *Drosophila* that have enriched our understanding of some forms of learning and memory, the role of context in learning and memory has not been extensively studied in flies. There are two studies that demonstrate the importance of visual context in fly learning and memory. In both these studies, a flight simulator was used in which a single fly, tethered to a copper wire, is suspended in a cylindrical arena. Recordings are taken as the fly flies and controls its position relative to the panorama that contains the desired visual stimuli (T-patterns of different orientation). Flies have been trained in an operantly or classically conditioned fashion to associate a chosen direction of flight (and the visual stimuli in that direction of flight) with heat (infrared), the US (Guo and Gotz, 1997). Liu et al. (1999) first studied the role of context, specifically context generalization, in a visual-learning paradigm using the flight simulator. The tethered fly received pairings of the reinforcer (heat, US) with certain orientations of flight (using T-patterns) in three contexts (CSs), white, monochromatic green or blue light contexts. During the total experiment time of 18 min in the flight simulator, pairing of heat and flight orientation occurred between minutes 4-8 and 10-14 and testing occurred between minutes 14-16. Learning was measured by the fly’s orientation in the presence of the contexts. Wild-type flies were able to show contextual learning in which memory was not observed when trained and tested in different color contexts, however, if the color context remained the same throughout the rest periods, training and testing, memory was observed. Flies were also
able to generalize color contexts and show memory when they were exposed to one color context during both training and rest periods and later tested in another color context during testing. They found that this context generalization was dependent on a brain structure, the mushroom bodies. Although mutant flies with smaller mushroom bodies and flies with ablated mushroom showed normal behavior and learning when trained and tested in the same context, they showed impaired context generalization when trained and tested in different contexts. Furthermore, the authors distinguished cue and context generalization by using different composite patterns as cues instead of changing the ambient light (context). They concluded that neither cue conditioning nor cue conditioning generalization were dependent on the mushroom bodies (Liu et al., 1999), only context generalization was.

In contrast to vertebrate literature, context conditioning or the facilitation of memory training and testing in identical contexts was not observed in flies (Liu et al., 1999); this could be a failure to associate context with the US or a successful adaptation of the fly brain to associate similar contexts (generalize) with the US. In a similar study, Brembs and Wiener (2006) confirmed that flies could show context generalization by pairing the context (color) and flight directions using heat as the US.

1.7 Context conditioning in *Chasmagnathus granulatus*

Context conditioning for habituation has been studied in the crab *Chasmagnathus*. The stimulus used in these studies is a rectangular overhead screen (the visual danger stimulus or VDS) that moves over the container holding the crab. Crabs respond to the VDS by immediately running away. An automated device containing the crab presents the VDS and measures the intensity and duration of the escape response (attempting to
run away). Habituation of the escape response to repeated presentations of the VDS has been parametrically characterized in the crab; both intensity and duration of the escape response decreases to an asymptotic level. When trained by presenting fifteen 9s VDS presentations (trials) for 45 min at a 171s inter-trial interval (spaced training), a persistent decrement in response to the VDS or long-term habituation to the VDS can be seen up to 5 days later (Lozada et al., 1990).

Short- and long-term habituation toward the VDS have also been observed in field studies of crabs living in their natural habitats (Fathala Mdel et al., 2010b; Fathala Mdel et al., 2010a). The escape response to the VDS is thought to be similar to the response produced by overhead predators (shorebirds) in its natural environment. Hermitte and Maldonado (2006) further characterized the VDS response by showing that the crab heart rate is altered throughout the duration of VDS habituation and remains altered for a period of time after VDS habituation. Hermitte and Maldonado (2006) show that memory retrieval of previous VDS presentations 24h later produces long-term physiological changes (heart rate); the authors suggest that VDS presentation may be a form of fear in the crab.

Pedreira et al. (1995) demonstrated context conditioning with habituation of the escape response in crabs. Crabs that were trained and tested 24h later in the presence of identical visual contexts (e.g. plain or black and white stripped bowl) show greater retention of habituation when compared to crabs trained and tested 24h later in different contexts. To show that associative learning of the context was occurring, latent inhibition (exposure to the context before presentation of the US) and extinction (exposure to the context between the training of the US and memory test) were shown to inhibit long-term
context conditioning (Maldonado et al., 1997). This association between the context and the features of the VDS can persist for a long time; over time, the escape response weakens and is replaced by a strong freezing response to the VDS; this freezing response is called context-signal memory (Tomsic et al., 1998). The context-signal memory is blocked by systemic cyclohexamide (a protein synthesis inhibitor) injections (Hermitte et al., 1999), positively regulated by angiotensins (Delorenzi et al., 1996), selectively regulated by a muscarinic cholinergic mechanism (Beron de Astrada and Maldonado, 1999), by the cAMP signal pathway (Romano et al., 1996; Locatelli et al., 2001; Locatelli et al., 2002; Locatelli and Romano, 2005), by the nuclear factor kB transcription factor (Freudenthal et al., 1998; Freudenthal and Romano, 2000; Merlo et al., 2002) and by the NMDA-like glutamatergic receptors (Pedreira et al., 2002; Troncoso and Maldonado, 2002).

The context-signal memory was used to study memory reactivation and the reconsolidation of memory process that immediately follows (Pedreira et al., 2002). On day 1, crabs were given the same training to form the context-signal memory. But on day 2 crabs were either exposed for 5 min to the context they were trained in or a novel context in which they did not receive any training; memory was reactivated by exposure to the context but not the US (VDS). Since reconsolidation is again sensitive to similar treatments that can block consolidation (Nader, 2003), a protein synthesis inhibitor, cyclohexamide, was administered to the crab at various time points during memory reactivation. On day 3, 24h after the reactivation session, crabs were tested with the presence of the VDS in the training context. Crabs that received reactivation preceded by (1h) or followed by (up to 4h) cyclohexamide in the same context as they were trained.
and tested in did not show context-signal memory. However crabs that received cyclohexamide during the same reactivation treatment, but in a novel context showed the context-signal memory. The authors concluded that crabs show context-dependent memory reconsolidation and that this reconsolidation process is protein- and NMDA receptor (NMDA receptor antagonist, MK-801)-dependent (Pedreira et al., 2002).

Recently, Suarez et al. (unpublished, cited in Perez-Cuesta and Maldonado, 2009) showed that during memory consolidation (4h after training) the context-signal memory can be updated to incorporate a novel context during later memory tests, and that this process was a result of the formation of a new memory trace and not the modification of the existing memory trace.

During memory reactivation (when the memory is recalled after the consolidation process), some studies have produced extinction of the previously consolidated memory while others have observed memory reconsolidation. Myers and Davis (2002) suggest that extinction and reconsolidation are competing and concurrent processes during memory reactivation; whether extinction or reconsolidation occurs is hypothesized to be regulated by the amount of time the CS is exposed for during memory reactivation. To reconcile conflicting findings, Pedreira and Maldonado (2003) used the context-signal memory consolidation procedure to study the mechanisms underlying extinction and reconsolidation during the reactivation session. First, it was shown that a 60min or more exposure to the context (CS) during reactivation sessions produced extinction, whereas context exposure for 45min or less during reactivation sessions produces memory reconsolidation. Next, they identified the time window for cycloheximide-dependent protein synthesis for extinction; cycloheximide injections 1h before or 1h after a 60min
exposure to the context during memory reactivation eliminated extinction of the context-signal memory. These findings support the CS time-exposure hypothesis during memory reactivation (Myers and Davis, 2002). In a recent study, Perez-Cuesta and Maldonado (2009) showed that, depending on the duration of the context (CS), the reconsolidation or extinction process is only triggered after the offset of the CS, and that reconsolidation and extinction processeses can occur simultaneously without interfering with each other.

Not only does the crab reveal various parametrics and mechanisms of context conditioning, but context conditioning has also been a useful tool in providing insight into theoretical models of learning and memory. Furthermore, the development of electrophysiological recordings of visual lobula giant neurons in intact wake animals offer good opportunities to study biologically meaningful visual stimuli at a neurophysiological level (Tomsic et al., 2003). For example, the activity of the lobula giant neurons decreases in response to repeated VDS presentations and this correlates with the intensity of the escape response (Medan et al., 2007; Sztarker and Tomsic, 2008).

1.8 Context conditioning in Caenorhabditis elegans

Over the last 20 years, *C. elegans* has developed into a powerful model system to study learning and memory. Rankin et al. (1990) was the first to study learning and memory in the worm in which habituation, dishabituation and long-term memory (24h) were demonstrated. With 302 neurons and a neural wiring diagram indicating approximately 5000 chemical and 3000 electrical synapses (White, 1986), it is possible to map behaviors to identified neurons and study sites of stimuli-induced neural plasticity. Furthermore, the worm’s genome has been mapped and sequenced facilitating gene
manipulations through forward and backward genetic screens, RNA interference techniques and various techniques to express genes at a specific time and/or locations during development.

Context conditioning in *C. elegans* was first demonstrated by Rankin (2000) in a well-characterized mechanosensory habituation paradigm. When mechanical taps (US) are delivered to the side of the Petri dish containing the worms, naïve worms usually swim backwards in response to the tap. Repeated presentations of taps will result in worms swimming backwards for progressively shorter distance until they reach a minimal/asymptotic level of reversal response. This habituation to tap has been well characterized; demonstrations of spontaneous recovery and dishabituation have supported the claim that the response decrement is due to habituation and not to motor fatigue or sensory adaptation.

In context conditioning, worms were trained (habituated) with 30 taps on either plain agar or in salt flavored agar (soluble sodium acetate), and then transferred onto plain agar plates (Rankin, 2000). One hour after training, worms were re-habituated (tested) on either a plain agar or sodium acetate flavored agar. Worms trained and tested on the same flavor (plain or sodium acetate) of agar plate showed greater retention (decreased reversal responses) of previous training compared to worms trained and tested on different flavored agar. Worms showed context conditioning when trained and tested at both a 10 and 60 second inter-stimulus interval (ISI). In order to show that the learning was due to an association between context and the US (tap), latent inhibition and extinction were demonstrated. In latent inhibition, worms that were exposed to the context 1h before receiving taps did not show context conditioning. Similarly in
extinction, worms that were left in the same context for the hour between training and testing did not show context conditioning (Rankin, 2000). This study demonstrated that *C. elegans*, with only 302 neurons, was able to show chemosensory context conditioning.

Other studies have demonstrated the role of context in *C. elegans* behavior using chemotaxis. Chemotaxis is the tendency for worms to move towards or away from specific chemical compounds. The widely used chemotaxis assay measures chemical “preference” by calculating the number of worms at a chemical location compared to the number of worms at a location with no chemical (Bargmann et al., 1993). Adaptation of the worm chemosensory system has been well characterized (Colbert and Bargmann, 1995). For example, when worms are exposed to a high concentration of an attractive chemical (taste or odor), subsequent transfer onto a chemotaxis assay reveals that worms are less attracted to the odor than worms that had never been exposed to that chemical.

Although in the laboratory worms are normally grown in the presence of food, *E. coli*, different behaviors are observed in the presence and absence of food contexts. Colbert and Bargmann (1997) showed that adaptation to the volatile odor benzaldehyde was altered in the presence and absence of food. Since dopamine transmission is implicated in sensation of food, Sawin et al. (2000) showed that dopamine regulates a slowing response when food-deprived worms are re-introduced to food. The food-regulated dopamine transmission is also involved in habituation to mechanosensory tap, a form of non-associative learning (Kindt et al., 2007). Worms habituate more rapidly in the absence of food than in the presence of food due to food-sensing dopaminergic mechanosensory neurons that modulate other mechanosensory neurons in the worm. These behaviors show that food contexts can regulate behaviors and subsequent learning
through dopamine neurotransmission.

In addition to chemical cues and the presence of food, internal states can also affect memory retention. Bettinger and McIntire (2004) showed that worms can associate an ethanol-induced intoxicated state with adaptation to an attractive odorant, benzaldehyde. In this behavioral assay, training by exposure to both benzaldehyde and ethanol resulted in inhibition of adaptation to benzaldehyde during the testing session only if intoxicating levels of ethanol was present during training. Pre-exposure to ethanol alone did not affect chemotaxis or adaptation to benzaldehyde. Worms with impaired dopamine neurotransmission *cat-1* and *cat-2* were unable to show this form of context conditioning, suggesting that dopaminergic signaling is critical for state-dependent adaptation in *C. elegans* (Bettinger and McIntire, 2004).

1.9 Summary

The findings from studies of context conditioning in invertebrates are consistent with many findings in vertebrates, suggesting that there is conservation in learning and memory mechanisms across phylogeny. Invertebrate studies have supported several key findings in context conditioning; the difference between cue and context conditioning, protein-dependence of long-term memory and the critical role of neurotransmission and electrical properties of the neural membrane in both context conditioning and other forms of learning and memory. The objectives of this thesis were to: 1) modify Rankin’s (2000) single worm assay for short-term taste context conditioning paradigm to short-term taste and smell context conditioning in a multiple worm assay, 2) show that short-term context conditioning for mechanosensory habituation is specific for taste and smell contexts in an effort to distinguish between genes responsible for detection of taste/smell and genes
responsible for learning and memory, 3) determine whether genes (glr-1 and nmr-1) involved in glutamate neurotransmission play a role in short-term context conditioning for mechanosensory habituation, 4) identify neuron(s) as possible sites of context (taste/smell)-tap (US) integration in which NMR-1 is required to show short-term chemosensory context conditioning for mechanosensory habituation, 5) demonstrate, for the first time, long-term associative memory in C. elegans by producing long-term memory for context conditioning for mechanosensory habituation, 6) test whether the long-term context conditioning for mechanosensory habituation is a conventional form of long-term memory, 7) determine whether genes (glr-1 and nmr-1) involved in glutamate neurotransmission also play a role in long-term context conditioning for mechanosensory habituation.
CHAPTER 2

2.1 Short-term context conditioning introduction

Although context conditioning is considered a type of classical conditioning, studies have shown that context and cue training with fear classical conditioning are mediated by different mechanisms. Depending on the nature of the cue/context and measurement of fear in rodents, contextual and cued fear conditioning are dependent on different brain regions within the amygdala and hippocampus (Holland and Bouton, 1999; LeDoux, 2000; Walker et al., 2005a). Additionally, rodent studies have also shown that different training procedures (massed and spaced; Scharf et al., 2002) and a gene (nNOS; Itzhak et al., 2010) differentially regulate context and cue conditioning. These dissections of context and cue conditioning have shown that these two forms of conditioning are distinct and separable forms of learning that are mechanistically distinct. Rankin’s (2000) observation of context conditioning for non-associative habituation in C. elegans offers an opportunity to investigate whether different cellular mechanisms contribute to associative and non-associative components of this form of learning in the nematode.

Rankin (2000) first demonstrated short-term chemosensory context conditioning of habituation to mechanical taps in C. elegans. Worms that were habituated and then rehabituated one hour later in the presence of a taste chemosensory contextual (sodium acetate) cue showed greater retention of memory for habituation when compared to animals trained and tested one hour later in different taste contextual cues. Demonstrating extinction and latent inhibition of context conditioning confirmed the associative nature of this form of learning (Rankin, 2000).

In C. elegans, Morrison et al. (1999) dissociated non-associative and associative
learning by showing that lrn-1 and lrn-2 mutant worms could show normal habituation to diacetyl (non-associative learning), but could not show associative learning when the diacetyl smell was paired with an aversive taste, acetic acid. However, lrn-1 and lrn-2 mutations are currently unknown and unmapped genes thus, do not advance our understanding of these processes. Using the same paradigm, a homolog of a glutamate receptor subunit, GLR-1 was also shown to be critical in both habituation to diacetyl and diacetyl/acetic acid associative learning (Morrison and van der Kooy, 2001). These studies show the feasibility of dissociating non-associative and associative learning in C. elegans. In this thesis I will extend these findings by further investigating mechanisms underlying non-associative and associative learning.

To aid in understanding the cellular mechanisms underlying chemosensory context conditioning, I developed protocols using two different sensory pathways, taste and smell, which will help me to distinguish between mutations that affect sensory pathways, and genes that are critical for learning. In these studies, I have modified Rankin’s (2000) single worm assay into a multiple (approximately 10 worms at a time) worm assay to increase throughput. My first hypothesis was that worms would show context conditioning to both taste and smell when conditioned with other worms in the multiple worm assay, and that mutations that disrupted one sensory pathway (taste or smell) would eliminate context conditioning for that sense, but leave context conditioning for the other sense intact.

2.2 General materials and methods for short-term context conditioning

Animals. Hermaphroditic C. elegans (Bristol wild-type N2, osm-3(pr802), odr-7(cx4), nmr-1(vm487) and glr-1(ky176) originally obtained from the Caenorhabditis
Genetics Center, University of Minnesota) were used in all experiments. They were maintained on 5-cm Petri plates filled with 10 ml nematode growth medium (NGM) agar and streaked with *Escherichia coli* (strain OP50; Brenner, 1974). All worm maintenance and experiments were carried out in a temperature (20 °C ± 0.5 °C) and humidity (35%-45%) controlled room.

**Outcrossing.** To ensure that other mutations in *nmr-1(vm487)* mutant worms were not affecting mechanosensory habituation, Tiffany Timbers (Timbers & Rankin, unpublished) outcrossed *nmr-1(vm487)* mutant worms into the lab N2 wild-type strain as described by Barriere and Felix (2005). Outcrossing decreases the number of other mutations in the worm.

**Generation of rescue strains and plasmid constructions.** To rescue NMR-1 in subsets of neurons in *nmr-1* worms, I injected plasmids containing NMR-1 under cell-specific promoters into the gonads of *nmr-1* worms. The plasmids for *pglr-1::nmr-1* and *ptdc-1::nmr-1* were received as gifts from V. Maricq (University of Utah, Salt Lake City, UT). Germ-line transformation was performed by microinjection as described by Mello et al. (1991). Briefly, a *myo-3p::gfp* was used as a co-injection marker in order to distinguish the progeny with the injected plasmid from worms that don’t. The DNA concentrations for the pPDDESt vector containing *pglr-1::nmr-1* and *ptdc-1::nmr-1* was 31 ng/µl and 22 ng/µl, respectively. The 10 µl injection mixture consisted of 4 µl of plasmid, 3 µl pUC19 (10pg/µl) and 3 µl *myo-3p::GFP* DNA (30 pg/µl), which resulted in the creation of *pglr-1::nmr-1*(vg72) and *ptdc-1::nmr-1* (vg75) mutant worms.

**Short-term context conditioning of habituation to tap.** To produce short-term mechanosensory habituation to mechanical taps to the Petri-dish were administered under a stereo-microscope (Wild M32, Wild Leitz, Heerbrugg, Switzerland), and recorded using a digital camera (Panasonic Digital 5100, Kadoma, Japan) and VCR (a Panasonic
AG1960, Kadoma, Japan). The VCRs were also used for scoring video-taped responses, and monitor (NEC) as shown in Figure 2.1. The tap stimuli were applied with an electrical device (Grass S88 stimulator, West Warwick, USA) that produced mechanical taps exerting 1-2 N of force to the side of the test plate. The Petri-dishes were placed in a plate holder attached to a micro-manipulator (Marzhauser model MM33, Wetzlar, Germany), that was used to keep the worm in the visual field of the microscope/video camera (Figure 2.1).

Figure 2.1 Experimental setup to produce tap stimuli. Worms on NGM agar-filled Petri plates are placed in a micromanipulator under a microscope. The metal wire is attached to a tapping device that receives pulses from the Grass 288 stimulator. A camera is mounted on the microscope to view and record the responses of the worm. After testing the worms, the recording will be played back and scored onto an acetate sheet; the acetate sheet will be computer analyzed.
For the context conditioning aspect of the training in each short-term context experiment, four-day old worms were divided into three groups based on the presence or absence of the chemosensory cue during training and/or testing. One group received the context cue for both training and testing, one group received the cue at just training and plain agar at testing and the third received the cue at testing and plain agar at training. When testing strains of mutant worms, a fourth group of wild-type worms with the context cue present at both training and testing was added to show that context conditioning was possible under the experimental conditions at that time. For all short-term context conditioning experiments, groups of ten worms were individually transferred to the training plate with a platinum wire pick and then given training consisting of 30 taps given at a 10s ISI. After training, the context was removed and worms were allowed to rest for a 1h period. After the rest period during testing, worms were given another 30 taps at a 10s inter-stimulus interval (ISI). Several replications were combined in each experiment.

*Multiple worm assay.* I tested approximately 10 worms at a time on an agar plate with one drop of *E. coli* in the middle of the plate. Each group in each experiment was made up of the sum of the data from 3-4 plates. For all experiments, 5-cm Petri plates were filled with 10ml NGM agar and seeded with 1 drop of OP50 (using a glass Pasteur pipette with bulb) one day before testing; this restricted the worms to a small area of the plate allowing for simultaneous recording of as many worms as possible at 10x magnification. When recording worm responses in the multiple worm assay, I focused on one group of worms during a tap (usually recording 4-7 worms at a time) and, after each tap, moved the plate to record different worms and to maximize the number of worms in
the field of view during VCR recording. Individual worms were not intentionally followed in this assay so this was not considered a repeated measures design. For statistical purposes, all response to the first two taps of training and the first two taps of testing were combined and analyzed to assess memory retention between training and testing sessions.

_Taste context conditioning._ For short-term taste context conditioning, the sodium acetate (C$_2$H$_3$NaO$_2$) context was created by washing seeded Petri plates with 400uL of 0.6 M sodium acetate solution, which was made daily. The plates washed with sodium acetate were allowed to dry for 1 h before testing began. After transferring worms onto the training plate, worms were allowed to recover for 3 min before training. Immediately after training, worms were transferred to a fresh NGM plate (containing _E. coli_) and then transferred onto the testing plate at the end of the 60 min rest period. For testing, worms were given 3 min to recover after they were placed on the test plate before administering the test 30 taps at a 10s ISI. For simplicity sake, the sodium acetate cue was shortened to NA in the figures and text and the plain agar condition was shortened to PL. Three groups were run for each taste conditioning experiment, a cue-cue (NANA) group, a cue-plain (NAPL) group and a plain-cue (PLNA) group.

_Smell context conditioning._ In short-term smell context conditioning, worms were transferred to the training plate and allowed to recover for 6 min. At the 4-minute mark the training plates and worms were brought into another room (to restrict any extraneous diacetyl odor to the set-up room and away from the test room) where a drop of diacetyl (shortened to DA) was placed in the center of the Petri-dish lid and the dish was sealed with parafilm to contain the smell; this was done for the cue-cue (DADA) and cue-plain
(DAPL) conditions. In the plain-cue (PLDA) condition, the worms were also taken into the set-up room, the lid was also opened, but no liquid was dropped onto the lid before it was parafilmed. After training, the plate of worms was taken back into the set-up room where a new lid with no smell was used and worms were allowed to rest for 1 h before testing (no parafilm). Before testing, a new lid with or without the odor was put onto the plate 2 min before testing began.

For all \( glr-1 \) and microinjected rescue mutant worms, mutant and wild-type control worms were transferred onto training/testing plates 1h before training. This was necessary because \( glr-1 \) worms were more sensitive to stimulation; pilot studies showed \( glr-1 \) worms had much smaller initial responses than wild-type worms when pre-plated for only 6min before taps, however, after 1h of pre-plating, \( glr-1 \) worm’s initial responses were not significantly different from responses of wild-type worms. For microinjected rescue transgenic worms, a 1h pre-plate was used due to the increased stimulation and time required for picking and transferring mutant worms using the fluorescence microscope (allows visualization of GFP) to distinguish the worms with and without the rescue construct.

Chemotaxis Assay for Diacetyl. To test whether the various mutant worms studied were able to detect and chemotax towards diacetyl, a chemotaxis assay was adapted from Bargmann and Horvitz (1991). The diacetyl smell was created by using a 1:100,000 mixture of diacetyl (Sigma, St Louis, USA) to ethanol (anhydrous ethyl alcohol, Commercial Alcohols Inc., Mississauga, Canada). This working solution was used for a maximum of 3 weeks and kept in the 4°C refrigerator. Working solutions were made from a stock of 1:100 solution of diacetyl to ethanol from the same fridge. Assay plates
were 10 cm Petri-dishes containing 30 ml of 1.6% NGM agar. Approximately 25 4-day-old worms were transferred by a platinum wire pick to an agar plate without food for one hour before testing with no more than 10 worms per 5cm agar plate to avoid excessive *E. coli* transfer onto the chemotaxis assay. During testing, drops of 1.5uL ethanol and liquid diacetyl (Sigma, St Louis, USA) were placed on the agar on opposite sides of the 10cm dish 0.5cm from the edge of the plate and 1.5uL drop of the paralytic sodium azide (1M; Sigma, St Louis, USA) was dropped 0.5cm from the ethanol and diacetyl drop in order to immobilize the worms at the target odor. This was immediately followed by the transfer of 25 worms onto the center of the plate, equidistant from the chemicals. The number of worms in the 1.5cm quadrant (from the edge of the region containing the diacetyl/ethanol) was counted every 10, 20, 30, 40, and 60 min. Both transfer and assays were carried out in a temperature and humidity controlled room unparafilmed and right side up. A chemotaxis index was calculated by subtracting the number of worms in the diacetyl quadrant from the number of worms in the control quadrant and dividing the difference by the total number of worms in the diacetyl and control quadrant (Bargmann et al., 1993).

**Data Analysis.** For all mechanosensory habituation studies, worm reversal responses to tap were traced using a VCR by hand onto an acetate sheet that was then scanned into an Apple iMac computer. The results were analyzed using Statview 4.5. Responses to tap were categorized into reversals (where the reversal length was traced onto an acetate sheet), ‘pauses’ (a stop after the tap stimulus for more than 1 second), a ‘no response’ (where the worm did not change behavior in response to tap stimulus), an ‘already reversing’ (where the worm was already reversing when the tap stimulus was...
given), or ‘acceleration’ (worm accelerates forwards at a velocity greater than 75% after tap stimulus). During data entry, a reversal was given a number value (based on the length of the reversal), a no response or pause was given a reversal length of zero and an ‘acceleration’ or ‘already reversing’ were entered as a null response (not calculated in statistical test and treated as missing data points). Although worms received 30 training taps and 30 test taps, not all were scored for analysis. Rankin (2000) showed that with a 10s ISI the context effect was largest in the first two responses of testing. My data showed this as well (Figure 2.2A) and so for all short-term context conditioning data, only the first two responses were scored and the average of the first two responses of training and testing were compared statistically. Response magnitudes were compared between groups using one-way ANOVA procedures. Fisher’s least significant difference was used for planned comparisons.

2.3 Results

2.3.1 Adult C. elegans show short-term context conditioning to taste and smell in a multiple worm assay

This experiment was designed to replicate the taste context conditioning for habituation that was reported by Rankin (2000) and extend it to smell context conditioning for habituation using a new higher throughput behavioral protocol that consisted of training and testing up to 10 worms at a time. As Rankin (2000) reported, the taste context conditioning facilitation of habituation was primarily observed in the first two taps, the third tap and subsequent taps were not significantly different between training and testing (Figure 2.2A). Therefore, like Rankin (2000), the average of the first two taps of training and testing were used to statistically assess context conditioning.
Figure 2.2 Wild-type worms showed short-term context conditioning to taste (NA) and smell (DA) in a multiple-worm assay. Wild-type worms showed short-term context conditioning to sodium acetate (NA) and diacetyl (DA) in which their training and testing responses to tap were analyzed. Context conditioning occurred if the testing responses to tap were significantly smaller than the training responses to tap. A) The NANA condition: Worms showed short-term context conditioning to NA in which their first two responses were significantly smaller. For Figure 2.2B) and 2.2C) bar graphs compare the average of the first two taps between training and testing; wild-type worms showed significantly smaller responses in the NANA/DADA condition when compared to PLNA/PLDA and NAPL/DAPL conditions (N\(\approx\)40 and N\(\approx\)35 per group, respectively). Error bars represent SEM and asterisks denote statistically significant differences. (F(5,289)=3.671, p\(\leq\)0.003; Figure 2.2B). Context conditioning was considered to have occurred when the mean of the first two responses to tap during testing was smaller than the responses during training (p\(\leq\)0.0031); this was only seen when trained and tested in sodium acetate (NANA condition; p\(\leq\)0.0002). There were no significant differences between the responses of training and testing when the worms were trained and tested in different contexts (PLNA or NAPL; p\(\leq\)0.76 and p\(\leq\)0.87, respectively). Thus, I replicated
Rankin’s (2000) context conditioning to taste with a multiple worm assay.

I also demonstrated that context conditioning of habituation occurred using smell (volatile diacetyl) as the context. When tested and trained in the presence of diacetyl (DADA condition), I observed similar results to context conditioning to taste; the responses to the first two taps were lower in testing compared to training (F(5,156)=3.042, p≤0.01; Figure 2.2C). In the DADA condition, the average of the first two taps was significantly lower in testing compared to training (p≤0.01). There were no differences between the responses of training and testing if the worms were trained and tested in different contexts (PLDA or DAPL; p≤0.40 and p≤0.89, respectively). Taken together, these data indicate that worms show both taste and smell context conditioning of habituation using multiple worm assays.

2.3.2 Context conditioning for mechanosensory habituation is specific for taste and smell contexts

To distinguish the chemosensory neurons responsible for detecting sodium acetate (taste) and diacetyl (smell) context conditioning I tested whether context conditioning is modality specific and operates through separable taste and smell pathways. To do this, I used worms with mutations in genes in either the taste or smell neural pathways. First, I tested osm-3 worms that were defective in taste (sodium acetate) chemotaxis (Bargmann et al., 1993); OSM-3 is a homodimeric kinesin motor protein required for intraflagellar transport and for formation of the distal segment of amphid channel cilia of taste-detecting chemosensory neurons (Shakir et al., 1993). I tested osm-3 worms and wild-type using both taste and smell context conditioning assays; osm-3 worms showed context conditioning to diacetyl (smell), but not to sodium acetate (taste). osm-3 worms
Figure 2.3 *osm-3* worms (taste defective) did not show short-term context conditioning to taste (2.3A) but did show context conditioning for smell (2.3B). All conditions were run with a wild-type (WT) NANA/DADA context condition at the same time and the average of the first two taps between training and testing were analyzed. A) *osm-3* worms did not show short-term taste context conditioning in any conditions (NANA, PLNA and NAPL); there were no significant differences between training and testing (N\(\cong\)21 per group). However, wild-type worms in the NANA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session. B) *osm-3* worms showed short-term smell context conditioning; in the DADA condition, the responses in the testing session were significantly smaller than in the training session, however, in the PLDA and DAPL conditions, the training and testing sessions were not significantly different from one another (N\(\cong\)25 per group). Similarly, wild-type worms in the DADA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session. Error bars represent SEM and asterisks denote statistically significant differences.

Trained and tested in the soluble sodium acetate cue did not show increased retention of mechanosensory habituation during testing when compared to wild-type worms (F(7,294)=2.761, p\(\leq\)0.009; Figure 2.3A); the average response of the first two taps during testing was not significantly lower than in training (p\(\leq\)0.44). There were no differences
between the responses of training and testing if the *osm-3* worms were trained and tested in different contexts (PLNA or NAPL; \(p \leq 0.17\) and \(p \leq 0.41\), respectively). In contrast, wild-type worms that were trained and tested in sodium acetate (NANA) had significantly smaller average responses during testing compared to the responses during training (\(p \leq 0.01\)).

On the other hand, in the test of smell context conditioning, *osm-3* worms trained and tested in the smell context conditioning paradigm with the volatile diacetyl cue did showed increased retention of mechanosensory habituation during testing when compared to wild-type worms; the average response of the first two taps during testing was significantly lower than in training (F(7,422)=3.246, \(p \leq 0.002\); Figure 2.3B). There were no differences between the responses of training and testing if the *osm-3* worms were trained and tested in different contexts (PLDA or DAPL; \(p \leq 0.80\) and \(p \leq 0.82\), respectively). Wild-type controls were run at the same time as mutant worms to show that experimental conditions were sufficient to produce context conditioning for mechanosensory habituation in controls. Similarly, wild-type worms that were trained and tested in diacetyl (DADA) had significantly smaller average responses during testing compared to the responses during training (\(p \leq 0.01\)).

In addition to demonstrating that taste defective *osm-3* worms showed context conditioning to smell and not taste, I also showed that smell defective *odr-7* worms showed context conditioning to taste and not smell. ODR-7 encodes for a nuclear receptor that affects the cell fate of the diacetyl-detecting AWA olfactory neurons, therefore *odr-7* worms cannot chemotax to diacetyl (Sengupta et al., 1996). *odr-7* worms trained and tested in the taste paradigm using the soluble sodium acetate cue showed
increased retention of mechanosensory habituation during testing when compared to 
wild-type worms (F(7,514)=2.626, p≤0.01; Figure 2.4A); the average responses of the 
first two taps during testing was significantly lower than in training (p≤0.004). There 
were no differences between the responses of training and testing if odr-7 worms were 
trained and tested in different contexts (PLNA or NAPL; p≤0.53 and p≤0.65, 
respectively). Similarly, wild-type worms that were trained and tested in sodium acetate 
(NANA) had significantly smaller average responses during testing compared to the 
responses during training (p≤0.02).

Figure 2.4 odr-7 worms (smell defective) showed short-term context conditioning to taste 
(2.4A), but not to smell (2.4B). Both conditions were run with a wild-type (WT) 
NANA/DADA context condition at the same time and the average of the first two taps 
between training and testing were analyzed. A) odr-7 worms showed short-term taste 
context conditioning; in the NANA condition, the responses in the testing session were 
significantly smaller than in the training session, however, in the PLDA and DAPL
conditions, the training and testing sessions were not significantly different from one another. Similarly, wild-type worms in the NANA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session. (N=31 per group). B) odr-7 worms did not show short-term smell context conditioning; in all the conditions (DADA, PLDA and DAPL) there were no significant differences between training and testing. However, wild-type worms in the DADA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session. (N=40). Error bars represent SEM and asterisks denote statistically significant differences.

Conversely, odr-7 worms trained and tested in the volatile diacetyl cue did not show increased retention of mechanosensory habituation during testing when compared to wild-type worms (F(7,581)=3.769, p≤0.0005; Figure 2.4B); the average responses to the first two taps during testing was not significantly lower than in training (p≤0.52). There were no significant differences between the responses of training and testing if the odr-7 worms were trained and tested in different contexts (PLDA or DAPL; p≤0.42 and p≤0.19, respectively). In contrast, wild-type worms that were trained and tested in diacetyl (DADA) had significantly smaller average responses during testing compared to the responses during training (p≤0.05).

This double dissociation shows that taste and smell context conditioning are mediated by different chemosensory neurons and allows me to identify genes specifically involved in the learning process rather than genes involved in the detection of sodium acetate (taste) or diacetyl (smell). With discrete taste and smell sensory pathways that could be used to show context conditioning, I began the search for genes that played a specific role in the associative aspect of context conditioning for mechanosensory habituation.

2.3.3 GLR-1 and NMR-1 are important for context conditioning in C. elegans

To investigate possible mechanisms for context conditioning for habituation, the
first genes I focused on were genes involved in glutamate neurotransmission because these genes have been shown to play important roles in non-associative long- and short-term habituation (Rose et al., 2003) and had also been shown to be involved in other types of associative learning (Morrison et al., 1999; Kano et al., 2008) in *C. elegans*. The glutamate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor subunit GLR-1 is a homologue of mammalian AMPA receptor type subunits and the N-methyl D-aspartic acid (NMDA) glutamate receptor subunit NMR-1 is a homologue of mammalian NMDA-type glutamate receptor subunits; both of these types of glutamate receptors are involved in associative learning and synaptic plasticity in mammals (Hart et al., 1995; Maricq et al., 1995; Brockie et al., 2001a; Brockie et al., 2001b). Morrison and van der Kooy (2001) found that *glr-1* worms did not show normal habituation to diacetyl (non-associative learning) or associative learning in which diacetyl odor was paired with an acetic acid taste. In *C. elegans*, Kano et al. (2008) found that NMR-1 was important for associative learning in which starvation was paired with sodium chloride taste. I hypothesized that GLR-1 and NMR-1 would not be required for short-term habituation to tap, but both GLR-1 and NMR-1 would be important for short-term chemosensory context conditioning to both taste and smell.

### 2.3.4 AMPA receptors are important for short-term smell context conditioning in *C. elegans*

To determine whether GLR-1 plays a role in associative learning, I tested worms with a mutation in the *glr-1* gene with smell context conditioning and compared their results with wild-type worms. First, I examined short-term habituation in *glr-1* and wild-type worms at a 10s ISI. As can be seen in Figure 2.5, worms with a mutation in GLR-1
showed initial responses to tap and short-term habituation at a 10s ISI that were indistinguishable from that of wild-type worms. I examined smell context conditioning using five groups (Untrained, Trained, DADA, DAPL and PLDA) of *glr-1* worms and a DADA context condition of wild-type worms. *glr-1* worms trained and tested with the volatile diacetyl cue did not show increased retention of mechanosensory habituation.

Figure 2.5 *glr-1* worms habituated normally at a 10s ISI, but did not show short-term olfactory context conditioning. A) There were no differences between the initial response to tap and habituation to tap for *glr-1* and wild-type (WT) worms in the first five taps (N≥50 per group). B) *glr-1* worms did not show short-term smell context conditioning; in all the conditions (DADA, DAPL and PLDA) there were no significant differences between training and testing. However, wild-type worms run at the same time showed context conditioning, the responses in the testing session were significantly smaller than in the training session. (N≥30 per group). Error bars represent SEM and asterisks denote statistically significant differences.
during testing ($F(7, 1736) = 2.348, p \leq 0.02$; Figure 2.5B); the average responses of the first two taps during testing for the glr-1 DADA group were not significantly lower than in training ($p \leq 0.34$). There were also no differences between the responses of training and testing if the worms were trained and tested in different contexts (PLDA or DAPL; $p \leq 0.08$ and $p \leq 0.83$, respectively). In contrast, the wild-type worms that were trained and tested (DADA) in diacetyl at the same time did have significantly smaller average responses during testing compared to the responses during training ($p \leq 0.009$).

**2.3.5 NMDA receptors are not critical for short-term habituation but are important for short-term smell context conditioning in C. elegans**

To determine the role of NMR-1 a glutamate NMDA NR1 receptor subunit homolog in context conditioning along with a wild-type DADA control in *C. elegans*, *nmr-1* worms were subjected to both taste and smell context conditioning. I observed that *nmr-1* worms showed significantly smaller responses to the first tap of training in both taste and smell conditioning (initial responses in the presences of the taste, sodium
acetate, and smell, diacetyl, for wild-type and \textit{nmr-1} worms were combined) than did wild-type worms (F(1,175)=16.852, p\leq0.0001; Figure 2.6). However, despite the lower initial responses, short-term habituation of \textit{nmr-1} worms in both taste and smell at a 10s ISI was very similar to that of wild-type worms, as can be seen in Figure 6.

I then tested \textit{nmr-1} worms with taste and smell context conditioning and found that they did not show either taste or smell context conditioning. \textit{nmr-1} worms trained and tested in the soluble sodium acetate cue did not show increased retention of mechanosensory habituation during testing when compared to wild-type worms (F(7,319)=2.423, p\leq0.01; Figure 2.7A); the average responses of the first two taps during the \textit{nmr-1} NANA testing was not significantly lower than in training (p\leq0.95). There were no differences between the responses of training and testing if \textit{nmr-1} worms were trained and tested in different contexts (PLNA or NAPL; p\leq0.28 and p\leq0.56, respectively). In contrast, the wild-type NANA worms that were trained and tested in sodium acetate (NANA) had significantly smaller average responses during testing compared to the responses during training (p\leq0.02).

\textit{nmr-1} worms trained and tested in the presence of the diacetyl cue did not show increased retention of mechanosensory habituation during testing compared to wild-type worms (F(7,478)=2.348, p\leq0.02; Figure 2.7B); the average responses of the first two taps during testing was not significantly lower than in training (p\leq0.10). There were no differences between the responses of training and testing if the \textit{nmr-1} worms were trained and tested in different contexts (PLDA or DAPL; p\leq0.81 and p\leq0.88, respectively). In contrast, wild-type worms that were trained and tested in diacetyl (DADA) at the same
Figure 2.7 *nmr*-1 worms did not show short-term context conditioning to taste (2.7A) or smell (2.7B). Both conditions were run with a wild-type DADA context condition at the same time and the average of the first two taps between training and testing were analyzed. Both wild-type worms showed context conditioning; the responses in the testing session were significantly smaller than in the training session. In both A) and B) *nmr*-1 worms did not show short-term taste and smell context conditioning in any conditions (NANA, PLNA and NAPL; N≈21 per group; DADA, PLDA and DAPL; N≈30 per group); there were no significant differences between training and testing. Error bars represent SEM and asterisks denote statistically significant differences.

time had significantly smaller average responses during testing compared to the responses during training (p≤0.01).

2.3.6 Rescuing NMR-1 in transgenic *nmr*-1 mutant worms

Kano et al. (2008) showed that, in *C. elegans*, the NMDA receptor subunit, NMR-
1, was critical for associating starvation with a taste (NaCl) in the RIM interneuron in *C. elegans*. The RIM interneuron is also a good candidate for the site of association in chemosensory context conditioning for mechanosensory habituation because it is one of afew neurons that connect the chemosensory neurons and the neurons involved in the mechanosensory circuit (White, 1986; Wicks and Rankin, 1995), as seen in Figure 2.8.

![Neural Circuit Diagram](image)

Figure 2.8 The neural circuit mediating tap response and chemosensation. *osm-3* is expressed in the ASE chemosensory neuron, which primarily detects sodium acetate. *odr-7* is expressed in AWA, which primarily detects diacetyl. RIM and AVA both express *nmr-1* and may be sites of plasticity in chemosensory context conditioning for habituation (*adapted from Sengupta, 2007; Tsalik and Hobert, 2003*).

To restore NMR-1 in transgenic *nmr-1* worms, Kano et al. (2008) expressed NMR-1 in transgenic *nmr-1* worms that expressed NMR-1 under the regulation of the *glr-1* promoter that drives expression in all cells that normally express NMR-1 as well as some additional cells that do not, as seen in Table 2.1. Kano et al. (2008) also rescued
*nmr-1* worms with a transgene that drives NMR-1 expression in just the RIM interneurons. I tested *nmr-1* worms carrying these two transgenes to see whether either or both of them rescued context conditioning in *nmr-1* worms. I hypothesized that NMR-1 would be required in only the RIM interneuron to show normal chemosensory context conditioning.

### 2.3.7 NMDA receptor subunits in the RIM interneurons are critical for short-term context conditioning

In order to further understand the role *nmr-1* was playing in context conditioning, I rescued NMR-1 in neurons that normally express NMR-1, including the RIM interneuron as well several additional neurons (Table 2.1). To do this, I expressed NMR-1 under the regulation of the *glr-1* promoter (*Pglr-1::NMR-1*) in *nmr-1* worms using the same construct (Kano et al., 2008) used to rescue salt-starvation associative learning in *nmr-1* worms. *Pglr-1::NMR-1* worms trained and tested in the volatile diacetyl cue did not show increased retention of mechanosensory habituation during testing when compared to wild-type worms (F(7,407)=6.064, p≤0.0001; Figure 2.9A); the average

<table>
<thead>
<tr>
<th>Gene</th>
<th>Neurons</th>
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<tbody>
<tr>
<td><em>glr-1</em></td>
<td>RIM AVA AVE AVG PVC AVD AVB AVJ PVQ RMD SMD URY</td>
</tr>
<tr>
<td><em>nmr-1</em></td>
<td>RIM AVA AVE AVG PVC AVD AVB</td>
</tr>
<tr>
<td><em>tdc-1</em></td>
<td>RIM (low expression in RIC)</td>
</tr>
</tbody>
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Table 2.1 Neurons that *glr-1*, *tdc-1* and *nmr-1* are normally expressed in. Neurons in bold font are the ones modeled in the chemosensory context conditioning for mechanosensory habituation neural circuit.
responses of the first two taps during testing were not significantly lower than in training (p≤0.8692). There were significant differences between the responses of training and testing if *Pglr-1::NMR-1* worms were trained and tested in different contexts. In the PLDA condition, worms trained in plain agar and tested in diacetyl had significantly lower average responses during testing (p≤0.02). In contrast, in the DAPL condition, worms trained in diacetyl and tested in plain agar had significantly lower average responses during training (p≤0.0001). However, wild-type worms that were trained and tested in diacetyl (DADA) had significantly smaller average responses during testing compared to the responses during training (p≤0.02). Examination of Figure 2.9A shows an interesting and novel pattern of results; all of the *Pglr-1::NMR-1* worms tapped in the presence of diacetyl showed smaller reversal responses than *Pglr-1::NMR-1* worms tapped without diacetyl (PL groups) and than wild-type worms in the presence of diacetyl. Examination of the first five responses (data not shown) of training and testing showed that *Pglr-1::NMR-1* worms consistently gave smaller responses to tap in the presence of diacetyl (DA) than they did when diacetyl was not present (PL condition). The *Pglr-1::NMR-1* transgene driven by the *glr-1* promoter did not rescue context conditioning for habituation and it altered the response to tap when diacetyl was present.

I also tested whether, as in a salt-starvation associative learning paradigm (Kano et al., 2008), expressing NMR-1 only in the RIM interneurons was sufficient to rescue smell context conditioning for mechanosensory habituation in transgenic *nmr-1* worms. To do this, I used *Ptde-1::NMR-1* worms that expressed NMR-1 under the regulation of the *tdc-1* promoter in *nmr-1* mutant worms; this promoter is specific for the RIM interneurons (Kano et al., 2008) hence NMR-1 would only be expressed in the RIM
Figure 2.9 Rescuing NMR-1 in the RIM (using Ptdc-1::NMR-1) interneuron restored olfactory context conditioning, however, using the Pglr-1::NMR-1 did not restore context conditioning. Both conditions were run with a wild-type (WT) DADA context condition at the same time and the average of the first two taps between training and testing were analyzed. A) Pglr-1::NMR-1 worms did not show short-term context conditioning to smell; responses between training and testing were not significantly different. Interestingly, responses in the PLDA and DAPL condition indicates that Pglr-1::NMR-1 worms have smaller responses when tapped in the presence of diacetyl, but not in plain NGM agar. However, wild-type worms in the DADA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session (N=26 per group). B) Ptdc-1::NMR-1 worms showed short-term smell context conditioning; in the DADA condition, the responses in the testing session were significantly smaller than in the training session, however, in the PLDA and DAPL conditions, the training and testing sessions were not significantly different from one another. Similarly, wild-type worms in the DADA condition showed context conditioning; the responses in the testing session were significantly smaller than in the training session. In addition, Ptdc-1::NMR-1 worms had a significantly smaller initial response to tap when compared to wild-type worms (N=33 per group). Error bars represent SEM and asterisks denote statistically significant differences.
interneurons. The *Ptdc-1::NMR-1* transgene did not rescue the small initial response in the *nmr-1* worms as the initial responses of *Ptdc-1::NMR-1* worms were significantly smaller than wild-type worms (*F*(1,83)=5.407, *p*≤0.02), however, the *Ptdc-1::NMR-1* transgene did rescue olfactory context conditioning. *Ptdc-1::NMR-1* worms trained and tested in the volatile diacetyl cue showed increased retention of mechanosensory habituation during testing when compared to wild-type worms (*F*(7.697)=3.363, *p*≤0.001; Figure 2.9B); the average response of the first two taps during testing was significantly lower than in training (*p*≤0.03). There were no differences between the responses of training and testing if *Ptdc-1::NMR-1* worms were trained and tested in different contexts (PLDA or DAPL; *p*≤0.31 and *p*≤0.93, respectively). Similarly, wild-type worms that were trained and tested in diacetyl (DADA) had significantly smaller average responses during testing compared to the responses during training (*p*≤0.04).

2.3.8 Chemotaxis to diacetyl for wild-type N2, *odr-7*, *glr-1* and *nmr-1* are not different from wild-type

To ensure that a deficit in detection or chemotaxis to diacetyl was responsible for deficits in olfactory context conditioning in the *glr-1* and *nmr-1* worms, these strains were tested using a diacetyl chemotaxis assay modified from Bargmann and Horvitz (1991). N2 wild-type controls, *glr-1* and *nmr-1* worms were able to chemotax to diacetyl, in which the three strains reached a chemotaxis index of 0.30-0.40 after 60 min, as seen in Figure 2.10. The line graph consisting of time points at 10, 20, 30 and 40 min for *glr-1*, *nmr-1* and N2 wild-type did not appear different from one another, however, the negative control, *odr-7* worms did show a deficit in chemotaxis to diacetyl in which *odr-7* worms remained at a chemotaxis index of 0-0.05 throughout the 60 min assay (Figure 2.10).
Figure 2.10 glr-1 and nmr-1 worms can successfully chemotax to diacetyl in a similar way to wild-type worms. The chemotaxis index for wild-type (N=75), odr-7 (N=60), glr-1 (N=80) and nmr-1 (N=70) worms observed and calculated at five time points after worms were transferred onto the chemotaxis assay plate at 10, 20, 30, 40 and 60 min. The negative control odr-7 (smell defective) did not chemotax toward diacetyl.

2.2 Discussion

Development of a new protocol. These results show that the new multiple-worm procedure for studying taste context conditioning in C. elegans replicated the results of the original procedure reported by Rankin (2000). Both paradigms produced context conditioning in which the context enhanced the retention of tap habituation during the first two test responses when trained and tested in the presence of the taste cue (sodium acetate). By using the new multiple-worm procedure instead of the single worm procedure, I can now assess short-term associative learning in days rather than weeks.

Rankin’s (2000) taste context conditioning procedure produced a larger effect between the initial responses between training and testing compared to the effect seen in
This study. This may be due to the presence of food (*E. coli*) in the multiple-worm procedure that was designed to keep worms in the microscope field of view. Previous studies have shown that the presence of food alters tap habituation kinetics (Kindt et al., 2007), locomotion (Sawin et al., 2000) and adaptation to volatile benzaldehyde (Colbert and Bargmann, 1997). Dopamine is implicated in these behavioral effects of food; this suggests that testing context conditioning for habituation in worms with mutations affecting dopamine-neurotransmission (*dop-1, cat-1*, etc…) might show enhanced context conditioning for mechanosensory habituation. It is also possible that the tastes and smells from food may interfere with the detection or salience of the sodium acetate taste in the agar.

The new group-training procedure for context conditioning was also effective when volatile diacetyl was used as a context cue. Consistent with many studies using olfactory cues in short-term associative learning in *C. elegans* (Morrison et al., 1999; Wes and Bargmann, 2001), worms showed context conditioning to smell with a similar pattern of memory retention to taste context conditioning, in which the context conditioning produced increased memory retention only in the first two responses. Having demonstrated associative learning for both taste and smell, this allows dissociation of taste and smell context conditioning in order to distinguish genes that are involved in the detection of taste and smell from genes that are involved in learning and memory.

*Genetic dissociation of taste and smell.* In support of my hypothesis, worms that cannot smell (*odr-7*) showed context conditioning to taste and not smell, conversely, worms that cannot taste (*osm-3*) showed context conditioning to smell and not taste. This
genetic dissociation of taste and smell confirms that taste and smell are detected through two different pathways in context conditioning for mechanosensory habituation. This allowed me to create a model neural circuit of taste and smell context conditioning, as seen in Figure 2.6. This model proposes candidate sites and neurotransmitters that may be important for integration and plasticity in response to environmental stimuli.

In *C. elegans*, researchers have associated taste, smell and temperature with the absence and presence of food (Wen et al., 1997; Mohri et al., 2005; Tomioka et al., 2006), however, no one has investigated associative learning using touch (mechanosensation). Adding taste and smell context conditioning for mechanosensation to existing associative learning assays will increase the likelihood of finding a gene whose general functions is associative learning if candidate worms are defective in a number of associative learning assays.

*Glutamate neurotransmission, GLR-1 and NMR-1, in context conditioning.* GLR-1 played a role in both taste and smell context conditioning; this was expected because glutamate neurotransmission was shown to be important for mechanosensory habituation (Rankin and Wicks, 2000) and olfactory associative learning in which a smell (diacetyl) was paired with an aversive taste (acetic acid; Morrison and van der Kooy, 2001). Consistent with the findings that NMR-1 is important for short-term associative learning in which starvation was paired with a sodium chloride taste (Kano et al., 2008), NMR-1 was also important for taste and smell context conditioning for mechanosensory habituation. These results support my hypothesis that glutamate neurotransmission, specifically GLR-1 and NMR-1, play a role in context conditioning for mechanosensory habituation.
Hoerndli et al. (2009) suggested that GLR-1 is important for olfactory and not gustatory context conditioning (Morrison and van der Kooy, 2001; Kano et al., 2008), in contrast I found that glr-1 worms could not show either taste or smell context conditioning for habituation. The possibility that GLR-1 plays a role in mechanosensory habituation leading to context conditioning deficits that are a result of deficits in habituation is unlikely because glr-1 worms show normal short-term habituation (Rose et al., 2003).

Testing genes implicated in non-associative and associative learning in taste and smell context conditioning will further allow me to dissociate between associative and non-associative learning. Some associative learning mutants investigated in previous studies include, ncs-1 (a neuronal calcium sensor), defective in associating food with temperature (Gomez et al., 2001), hen-1 (a secreted protein that containing a low-density lipoprotein receptor motif), defective in associating food with temperature (Chi et al., 2007) and casy-1 (a transmembrane protein with two extracellular cadherin domains), defective in associating food with temperature and the taste sodium chloride (Hoerndli et al., 2009). CASY-1 was first isolated in a screen for genes involved in human cognition (Papassotiropoulos et al., 2006). Hoerndli et al. (2009) suggest that CASY-1 acts in the GLR-1 pathway; since GLR-1 is critical in context conditioning, CASY-1 is likely to play a role in both taste and smell context conditioning. To find genes specific for non-associative learning, I can test tap habituation mutants recently identified in a tap habituation-defective mutant screen (Giles and Rankin, unpublished); this will offer an opportunity to identify mutants that cannot show non-associative learning (tap habituation), but have intact associative learning (context conditioning).
Alternatively, to discover new genes involved in chemosensory context conditioning for mechanosensory habituation, a candidate gene approach for a genetic screen can be run using a newly developed ultra-high throughput multi-worm tracker for tap habituation (Rankin, personal communications) to identify novel genes involved in olfactory context conditioning. Genes identified in this screen can also be tested in a number of other associative learning paradigms and can be tested in non-associative learning paradigms such as habituation to tap and adaptation to tastes and smells to further dissociate between non-associative and associative learning.

_NMR-1 rescues._ As predicted from examination of the neural circuit for context conditioning for mechanosensory habituation, NMR-1 in the RIM interneurons (Pt
cdc-1::NMR-1) was critical for olfactory context conditioning. This is consistent with Kano et al.’s (2008) study, in which they found that short-term taste associative learning was also dependent on NMR-1 in the RIM interneurons. These studies suggest that the RIM interneurons are sites of integration for taste, smell and tap stimuli.

To understand the cellular changes during integration of different stimuli, investigating the cellular changes from individual stimuli vs. simultaneous stimuli in chemosensory context conditioning may be fruitful. One way to do this is to use transgenic mutant worms with combined _in vivo_ optical stimulation of the neuron (with a genetically encoded channel rhodopsin) with simultaneous calcium imaging (with a genetically encoded calcium indicator) to measure calcium levels during stimulation (Guo et al., 2009). Since worms have already been generated for _in vivo_ stimulation and calcium recording in the RIM interneuron (Guo et al., 2009), this will allow me to visualize how calcium signaling differs in context conditioned and naïve worms. In
vertebrates, NMDA receptors (homologs of NMR-1) regulate calcium influx, which alters subsequent behavior in the short- and long-term (Corrigan et al., 2005). Since NMDA receptors are also important in short-term context conditioning in *C. elegans*, lasting changes in calcium signaling after training is expected.

To restore context conditioning behavior in transgenic *nmr-1* mutants, expression of NMR-1 under the regulation of the *glr-1* promoter driving expression in cells that normally express NMR-1 did not produce olfactory context conditioning. Instead, there was an interesting effect in which *Pglr-1::NMR-1* worms that received taps in the presence of diacetyl had significantly smaller responses compared to *Pglr-1::NMR-1* worms in plain agar. This could have been caused by using the *glr-1* promoter, which drives expression in a larger subset of interneurons (see Table 2.1) than endogenous *nmr-1* expression. The observed responses might also be due to the expression of NMR-1 in AVB (command interneuron for forward locomotion), an interneuron that does not normally express NMR-1. Alternatively, expressing NMR-1 by microinjection (used in this study) that causes over-expression of NMR-1 under the *glr-1* promoter, may alter the membrane kinetics of command interneurons (AVA, AVB, AVD and PVC; (Brockie et al., 2001b), and in turn alter the tap withdrawal response when these cells respond to both diacetyl and tap. The role of NMR-1 expression under the *glr-1* promoter may be clarified by testing whether *Pglr-1::NMR-1* mutants show a similar effect with taste context conditioning. In addition, I plan to test whether rescuing *nmr-1* with its own endogenous promoter shows the same effect.

It is evident that both *Ptdc-1::NMR-1* and *Pglr-1::NMR-1* worms can detect diacetyl since *Ptdc-1::NMR-1* worms can show context conditioning using diacetyl as a
context cue and Pglr-1::NMR-1 worms show decreased tap withdrawal response only in the presence of diacetyl. However, it will be interesting to see if they chemotax towards diacetyl normally. Since NMR-1 is being over-expressed in various command interneurons for locomotion in both strains, it may be that chemotaxis to diacetyl is affected in both Ptde-1::NMR-1 and Pglr-1::NMR-1 worms.

This is the first study to show associative learning to mechanosensation in C. elegans and the genes important for learning this are consistent with other genes implicated in associative learning in other behavioral assays. Dissociating two modalities (taste and smell) in the multiple-worm procedure for context conditioning for mechanosensory habituation provides a unique opportunity to identify learning and memory mutants and distinguishes non-associative habituation from associative context conditioning. The data from these experiments has verified a site of plasticity (RIM interneurons) and offers an in vivo system to study glutamate neurotransmission in the integration of different stimuli that results in behavioral changes.
CHAPTER 3

3.1 Long-term context conditioning introduction

In the first set of experiments in this thesis, short-term memory for chemosensory context conditioning of habituation was studied. In this second set of experiments, I investigated whether *C. elegans* is capable of long-term memory for context conditioning of habituation. Long-term memory for context conditioning has been shown in a number of invertebrates and vertebrates. In humans, Herz (1997) found that olfactory context conditioning enhanced memory for a list of words when both learning and free-recall occurred in the presence of the same distinctive olfactory cue (osmanthas). In invertebrates, Pedreira et al. (1995) found that crabs can associate visual stimuli on the walls of their training chambers (e.g. stripes, different lighting, etc…) with long-term habituation produced by spaced training with repeated presentations of an aversive visual US; this was demonstrated by increased retention of habituation to the US when crabs were trained and tested in the same visual context (Pedreira et al., 1995).

In *C. elegans* non-associative long-term memory for habituation to tap can last at least 48h depending on the protocol of tap presentation during training. Worms trained in a long-term memory spaced training procedure showed a decreased response to tap 24h and 48h after training compared to untrained worms or worms that were trained in a massed training procedure in which the same number of taps were presented (Beck and Rankin, 1995; Rose et al., 2003). *C. elegans* can also show several types of short-term associative learning; they can associate the presence/absence of food with specific temperatures, odors and tastes (Wen et al., 1997; Mohri et al., 2005; Tomioka et al., 2006). In these associative learning paradigms, long-term associative memory has not been studied in any depth because most of these learning procedures associate.
conditioned stimuli (CS) with the presence/absence of food (US). The issue with using food lies in the worms’ behavioral, physiological and cellular responses to starvation (stress; Suo et al., 2006; Angelo and Van Gilst, 2009; Kang and Avery, 2009); these starvation responses could easily confound or interfere with processes underlying learning and memory.

I modified the short-term olfactory context conditioning procedure developed in Chapter 2 and combined it with a spaced training procedure that produces long-term habituation (Rose et al., 2002) to determine whether context conditioning would facilitate long-term memory for habituation in C. elegans. These studies used olfactory and not taste context cues because context conditioning to taste requires moving worms from plate to plate; this would disrupt long-term memory for mechanosensory habituation (Rankin, personal communication). Furthermore, the neural circuit for taste is not as well understood as the neural circuit for smell (Bargmann et al., 1993). Therefore, in all long-term context conditioning experiments, I used the volatile odorant diacetyl in a smell context conditioning paradigm. My first hypothesis was that the presence of an olfactory context cue during both training and testing would enhance long-term memory for mechanosensory habituation.

3.2 Materials and methods for long-term context conditioning

Animals. Hermaphroditic C. elegans (Bristol wild-type N2, nmr-1(vm487), glr-1(ky1761) and crh-1(yt17) originally obtained from the Caenorhabditis Genetics Center, University of Minnesota) were used in all experiments. They were maintained on 5-cm Petri plates filled with 10 ml nematode growth medium (NGM) agar and streaked with Escherichia coli (strain OP50; Brenner, 1974). All worm maintenance and experiments
were carried out in a temperature (20 °C ± 0.5 °C) and humidity (35%-45%) controlled room.

*Long-term context conditioning.* For long-term smell context conditioning, four-day old worms were transferred onto NGM plates seeded with one drop of *E. coli*. Instead of using taps to the side of the Petri plates as in the short-term context conditioning, I stimulated worms with mechanosensory stimulation from box-drops so I could train several plates of worms simultaneously. For box-drops, the Petri-dishes containing the worms were placed into a 40cm by 30 cm Tupperware plastic box and dropped onto a counter from a height of 10cm; this was the US. This allowed several plates of worms to be trained at once.

To test whether long-term memory for habituation could be enhanced with the olfactory context conditioning procedure, worms were given 4 blocks of 20 box-drop stimulation at either a 60s or 10s (Figure 3.1A and 3.1B, respectively) with a 1h rest period between blocks. The context was manipulated by changing the lids (treated with nothing or diacetyl) before and after each block of training. An additional experiment was run using a massed training procedure in which the same number of stimuli (80 box-drops) were given consecutively at a 60s ISI and tested 24h later (Figure 3.1C). In every experiment, there were 5 groups, DADA (trained and tested in diacetyl), DAPL (trained in diacetyl and tested in a plain plate), PLDA (trained in a plain plate and tested in diacetyl), as well as a trained and untrained control group (in which no diacetyl was presented and lids were sealed during training until testing). The untrained control group received only a single box-drop on day one. As in the short-term context conditioning procedure, the PLDA and DAPL groups had their lids removed and were re-parafilmed.
Figure 3.1 Long-Term Olfactory Context Training Procedures. Worms were trained in the presence of diacetyl (DA) and tested 24h later in either plain NGM agar (PL; DAPL) or in diacetyl (DADA). Only the DADA and DAPL conditions are shown here. A) Spaced training in which 20 taps at a 60s ISI were presented during each of the four blocks of training with 1h rest periods between each block of training. B) Spaced training in which 20 stimuli at a 10s ISI were presented during each of the four blocks of training with 1h rest periods between each block of training. C) Massed training in which 80 stimuli at a 60s ISI were presented during one block. Even in the PL (plain agar, no cue) conditions, the trained and untrained control groups remained parafilemed throughout training and testing with no change in context.

Testing occurred 24h (+/- 4 h) after training and consisted of 10 taps at a 10s or 60s ISI in either the DA or PL conditions. During testing, worms were tested with tap in the same multiple worm assay as described in Chapter 2. In brief, I focused on one group
of worms during a tap (usually recording 4-7 worms at a time) and moved the plate to record different (sometimes overlapping) groups of worms after each tap to maximize number of worms in the field of view during VCR recording. Individual worms were not intentionally followed in this assay so this was not considered a repeated measures design. For statistical purposes, responses to the first two taps in the case of a 10 s ISI and responses to the first five taps in the case of a 60 s ISI were combined and analyzed to assess memory retention between training conditions (DADA, PLDA and DAPL).

Chemotaxis assay for diacetyl. To ensure mutant worms would detect and chemotax towards diacetyl, a chemotaxis assay was adapted from Bargmann and Horvitz (1991) as described in Chapter 2.

Data Analysis. Behavioral scoring and analyses of the test responses were carried out in this study the same way as in the short-term chemosensory context conditioning study. When test taps are delivered at a 60s ISI the response decrement is slow (Rankin and Broster, 1992) and so either the first five or ten taps have been traditionally used to assess long-term memory for habituation (Rose et al., 2003). The convention of five test taps has been followed here for stimuli delivered at a 60s ISI. When test taps were delivered at a 10s ISI, the decrement is much more rapid and so, as in the short-term context conditioning for habituation at a 10s ISI in Chapter 2, only the first two test responses were used to assess memory for context conditioning when habituation was at a 10s ISI. A significant difference between the untrained control group and any of the trained groups was considered evidence for long-term memory for habituation. A significant difference between the DADA group and the trained groups (Trained, DAPL and PLDA) was considered evidence for context conditioning. To assess context
conditioning, the average of the first responses for each group (Untrained, Trained, DADA, DAPL and PLDA) were compared using an ANOVA with Fisher’s least significant difference used for all planned comparisons.

3.3 Results

3.3.1 *C. elegans* can show long-term olfactory context conditioning for habituation

In *C. elegans*, long-term memory for habituation has been observed when worms receive spaced training: four blocks of 20 stimuli at a 60s ISI with a one hour rest period between each block (Beck and Rankin, 1995). To see whether olfactory (diacetyl) contextual cues enhanced long-term memory for habituation, I presented the worms with the diacetyl context cue during each block of 20 stimuli and not during the inter-block intervals. When the first five taps were averaged, the untrained control group that did not receive spaced training had significantly higher reversal responses to tap compared to the other four conditions (Trained, DADA, PLDA, DAPL) that received spaced training with and without diacetyl ($F(4,670)= 4.874, p \leq 0.0007$; Figure 3.2). This finding is consistent with previous findings for long-term habituation (Rose et al., 2002), and shows that the extra stimulation caused by repeated lid changes, diacetyl exposure(s), and exposure to lab air does not affect memory for spaced-training in long-term habituation. Because context conditioning was seen only in the first two taps in short-term olfactory context conditioning at a 10s ISI in the previous experiments, I also averaged the initial two test responses for each group and found no significant difference between groups and thus, regardless of the number of stimuli examined, there was no evidence for long-term memory for habituation or context conditioning (data not shown).

Context conditioning did not enhance long-term memory for habituation in the
Figure 3.2 Spaced training at a 60s ISI does not produce long-term context conditioning. In wild-type worms, no long-term olfactory context conditioning was observed; the Untrained condition had significantly higher reversal responses to tap compared to all other groups (Trained, DADA, PLDA and DAPL; N=30 per group). Error bars represent SEM and asterisks denote statistically significant differences.

The usual 60s ISI spaced training protocol, therefore in the next experiment I tested whether context would lead to enhanced memory for a training procedure that does not normally produce memory 24h after training. In this training procedure, worms were given the same four block spaced-training as above, however within each block, stimuli were delivered at a 10s instead of a 60s ISI. When the means of the first two taps were averaged F(3,414)= 2.276, p≤0.08; Figure 3.3A), the overall ANOVA was not significant, however because I was interested in specific comparisons, I am reporting the planned comparisons. Only the first two reversal responses during testing in the DADA condition were significantly lower than the Untrained (p≤0.05) and DAPL condition (p≤0.0147). These data suggest that context cues can produce memory after training that would not normally lead to long-term memory in C. elegans. Further, these findings indicate that worms are capable of associating diacetyl with habituation to tap 24h after training with stimuli at a 10s ISI.
Figure 3.3 Spaced training at a 10s ISI and not massed training at a 60s ISI produced long-term context conditioning. A) In wild-type worms, spaced training at a 10s ISI produced olfactory context conditioning in which the DADA condition was significantly different for the Trained and DAPL but not from the Control condition (N≅45 per group). B) In wild-type worms, massed training at a 60s ISI did not produce long-term olfactory context conditioning in which all conditions were not significantly different from one another (N≅22 per group). Error bars represent SEM and asterisks denote statistically significant differences.

To investigate whether olfactory context conditioning would produce memory in another protocol that does not normally produce memory, a massed training procedure. I adapted the olfactory context conditioning procedure to a 60s ISI massed training procedure. In this experiment, the context cue (diacetyl) was present at either training or testing or both. Here, wild-type worms (Trained, DADA, PLDA and DAPL) were
presented with 80 box-drops at a 60s ISI and tested with five taps 24h later (F(4,847)=1.244, p≤0.29; Figure 3.3B). None of the conditions (Untrained, Trained, DADA, PLDA and DAPL) were significantly different from one another when the first five taps were averaged. This replicates previous findings in which massed training at a 60s ISI did not produce memory for habituation 24h after training (Beck and Rankin, 1997). These data suggest olfactory context conditioning cannot enhance memory in all training procedures and that memories produced by different training procedures may have different underlying mechanisms.

3.3.2 Role of CRH-1 in olfactory long-term context conditioning for mechanosensory habituation

Having established that an olfactory cue could enhance memory for habituation after spaced training at a 10s ISI, I tested several mutant strains of worms to investigate possible mechanisms underlying this memory. By convention long-term memory depends on gene transcription. One molecule implicated in memory-gene transcription in many animals is the transcription factor cAMP response element-binding (CREB; Yin and Tully, 1996). In *C. elegans* the homolog of CREB is CRH-1. Worms with mutations in *crh-1* do not show long-term memory for habituation at a 60s ISI (Timbers and Rankin, Unpublished). Since, pilot studies indicated that *crh-1* worms showed normal short-term olfactory context conditioning with habituation at a 10s ISI, I tested *crh-1* worms in the long-term olfactory context conditioning protocol, to test whether the context enhancement of long-term memory for habituation was gene transcription-dependent. I hypothesized that worms with a mutation in *crh-1* would not show context dependent long-term habituation.
To enhance the olfactory context conditioning effect and to increase efficiency by lowering the number of animals needed to observe olfactory context conditioning, subsequent experiments with mutations that might affect context conditioning used six blocks of spaced training at a 10s ISI instead of four blocks.

To test whether the long-term olfactory context conditioning procedure is a protein synthesis-dependent form of long-term memory, I tested *crh-1* mutant worms lacking CREB, a transcription factor critical for long-term memory in many different organisms (Yin and Tully, 1996). In a comparison of the initial responses, *crh-1* mutant worms had significantly smaller responses to tap compared to N2 wild-type worms across conditions (F(1,234)= 4.596, p≤0.03; data not shown); this suggests that *crh-1* may play a role in the tap withdrawal reversal response. In other work, Timbers and Rankin

![Graph](image)

Figure 3.4 *crh-1* worms did not show long-term context conditioning. *crh-1* worms did not show long-term olfactory context conditioning however, wild-type worms run at the same time did. All conditions the responses of *crh-1* worms were not significantly different from one another (N=23 per group), whereas the DADA condition in wild-type worms was significantly lower than all conditions except for PLDA condition (N=24 per group). Error bars represent SEM and asterisks denote statistically significant differences.
unpublished) have shown that *crh-1* worms had normal short-term habituation. In long-term context conditioning, as controls, I ran comparable groups of wild-type worms at the same time as the *crh-1* worms. Worms with a mutation in *crh-1* showed neither long-term memory for habituation nor long-term context conditioning; a two way ANOVA showed a main effect of strain (F(1,437)=18.650, p≤0.0001) but not of condition (F(1,437)=2.040, p≤0.09) and no interaction of strain x condition (F(4,437)= 1.518, p≤0.20). Planned comparisons showed that the average first two responses were not significantly different between any of the five *crh-1* conditions, Untrained, Trained, DADA, PLDA and DAPL when compared to wild-type controls (Figure 3.4). However, the wild-type controls did show olfactory context conditioning; the wild-type DADA context condition had significantly lower responses compared to the Untrained (p≤0.04),

Figure 3.5 *crh-1* worms show normal chemotaxis to diacetyl. *crh-1* worms show normal chemotaxis to diacetyl (N≥50) when compared to wild-type worms(N≥75); both strains reach the same chemotaxis index after 60 min in the chemotaxis.
Trained (p≤0.02) and DAPL (p≤0.004) conditions, but was not quite significantly lower than the PLDA (p≤0.08) condition. To confirm that crh-1 worms could detect the diacetyl cue, a chemotaxis assay to diactyl given to crh-1 mutant worms indicated that they chemotaxed to diacetyl initially more slowly, but had a similar chemotaxis index when compared to wild-type worms after 60min (Figure 3.5).

### 3.3.3 Long-term olfactory context conditioning is dependent on NMR-1 and GLR-1

Because my earlier experiments indicated that short-term olfactory context conditioning was dependent on both GLR-1 and NMR-1, I tested the role of these two genes in long-term olfactory context conditioning for mechanosensory habituation. Rose et al. (2003) showed that glr-1 worms did not show long-term memory for habituation when spaced training was at a 60s ISI, however, nmr-1 worms did (Rose and Rankin, 2003). Figure 3.6 glr-1 worms did not show long-term context conditioning. glr-1 worms did not show long-term olfactory context conditioning however, wild-type worms run at the same time did. All conditions in which the glr-1 worms were run were not significantly different one another (N=29 per group), whereas the DADA condition in wild-type worms was significantly lower than all conditions except for DAPL condition (N=30 per group). Error bars represent SEM and asterisks denote statistically significant differences.
unpublished). I hypothesize that GLR-1, NMR-1 and CRH-1 will also be critical for long-term olfactory context conditioning in *C. elegans*.

In olfactory context conditioning for habituation, a two way ANOVA on data from *glr-1* and wild-type worms did not show a main effect of strain (F(1,640)=2.288, p≤0.13), showed an effect of condition (F(1,640)=2.353, p≤0.05) but no interaction of strain x condition (F(4,640)=1.089, p≤0.36). Planned comparisons showed that *glr-1* worms showed no significant differences between the average of the first two test responses for Untrained, Trained, DADA, PLDA and DAPL conditions (Figure 3.6). However, the wild-type controls run at the same time did show olfactory context conditioning; the DADA wild-type condition had significantly lower responses compared to the Untrained (p≤0.01), Trained (p≤0.009) and PLDA (p≤0.003) wild-type conditions,

![Graph](image.png)

Figure 3.7 *nmr-1* worms did not show long-term context conditioning. *nmr-1* worms did not show long-term olfactory context conditioning however, wild-type worms run at the same time did. All conditions in which the *glr-1* worms were run were not significantly different one another (N=40 per group), whereas the DADA condition in wild-type worms was significantly lower than all (N=45 per group). Error bars represent SEM and asterisks denote statistically significant differences.
but was not quite significantly lower than the DAPL (p ≤ 0.06) wild-type condition. Similarly, in \textit{nmr-1} worms did not show long-term context conditioning; a two way ANOVA showed a main effect of strain (F(1,655) = 15.748, p ≤ 0.0001) and of condition (F(1,655) = 2.536, p ≤ 0.04) but not of strain x condition (F(4,655) = 1.163, p ≤ 0.33). In a planned comparison, there were no significant differences between the averages of the first two test responses for the \textit{nmr-1} mutant Untrained, Trained, DADA, PLDA and DAPL conditions when compared to wild-type worms (F(9,646) = 3.644, p ≤ 0.0002; Figure 3.7). Once again the wild-type controls showed olfactory context conditioning; the DADA wild-type condition had significantly lower responses compared to the Untrained (p ≤ 0.01), Trained (p ≤ 0.03), PLDA (p ≤ 0.03) and DAPL (p ≤ 0.002) conditions.

3.4 Discussion

In this study, a novel behavioral assay was developed to show long-term associative learning in \textit{C. elegans} by modifying short-term olfactory context conditioning training procedures to test memory in the long-term context. The addition of an olfactory cue to an intermediate-term training procedure (10s ISI spaced training) produced long-term memory 24h later; our results are consistent with Beck and Rankin’s (1997) findings in which 10s ISI did not produce long-term memory when the olfactory cue was not present in both training and testing. These data support the hypothesis that context conditioning can enhance memory in \textit{C. elegans}.

Consistent with previous studies (Rose et al., 2002), spaced training at a 60s ISI produced long-term memory for habituation 24h later. However, in contrast to my hypothesis, the addition of a context cue in both training and testing did not enhance habituation retention 24h later. This may be due to a floor effect of memory retention for
habituation or fundamentally different mechanisms underlying habituation at a 10s ISI spaced training and habituation at a 60s ISI spaced training) as suggested by Broster and Rankin (1994) and Beck and Rankin (1997).

To verify that long-term olfactory context conditioning for mechanosensory habituation is a conventional protein synthesis-dependent form of long-term memory, we showed that *crh-1* worms lacking CREB did not show long-term olfactory context conditioning. Although CREB (a transcription factor) is critical for long-term memory in many different organisms (Yin and Tully, 1996), it is possible that the ubiquitous expression of CREB in the worm affects another behavior required to express long-term memory for context conditioning rather the memory consolidation process. Hence, it will be crucial to show converging lines of evidence that this long-term olfactory context conditioning is a form of long-term memory. Rose et al. (2003) showed that heat shock (inhibits protein synthesis) blocked long-term memory for tap habituation, so it is likely that heat shock presented in a similar fashion (during the rest period between blocks of training) would eliminate long-term memory for olfactory context conditioning. Another way to show protein synthesis-dependence of long-term olfactory context conditioning is drug treatment of cycloheximide; cycloheximide blocks context conditioning for habituation in the crab (Pedreira et al., 1995) and long-term associative learning in a number of other organisms (DeZazzo and Tully, 1995). Cycloheximide has been studied in *C. elegans*; low doses of cycloheximide is an attractant (Tajima et al., 2001) and pilot studies have shown that it blocks long-term memory for habituation at a 60s ISI (Timbers and Rankin, unpublished). Testing whether cycloheximide will block long-term context
conditioning of habituation at a 10s ISI will confirm that this memory is protein synthesis-dependent.

The memory deficit in *crh-1* worms also suggests that long-term olfactory context conditioning is unlikely a form of intermediate-term memory; ongoing research showed that *crh-1* worms that received both spaced and massed training had normal intermediate-term memory when tested 12h after training (Timbers and Rankin, unpublished). To further test if CREB plays a role in long-term memory for olfactory context conditioning of mechanosensory habituation, over-expression of CREB in extra-chromosomal arrays may enhance long-term context conditioning if this memory molecule functions in the same way in *C. elegans* as it does in other animals (Yin et al., 1995; Yin and Tully, 1996; Moncada and Viola, 2006). Over-expression of CREB may also lead to the expression of enhanced long-term memories in massed training and spaced training at a 60s ISI. In worms with mutations in CREB, rescuing CREB in the RIM interneuron under the regulation of the RIM-specific *tdc-1* promoter may restore long-term memory for olfactory context conditioning and confirm the critical role of RIM interneurons in associative learning. With several components identified to play a role in long-term olfactory context conditioning, a molecular pathway can now be investigated.

Since Chapter 2 showed that GLR-1 and NMR-1 played a role in short-term context conditioning for mechanosenosory habituation, it is not surprising that GLR-1 and NMR-1 also played a role for long-term olfactory context conditioning. NMR-1 was important for long-term associative learning for tap habituation, however was not important for non-associative long-term habituation (Rose & Rankin, unpublished); this is an elegant dissociation between long-term non-associative and long-term associative
learning. Brockie et al. (2001b) showed that the function of GLR-1 is not dependent on NMR-1 (NMDA-gated currents) and that although NMR-1 and GLR-1 are expressed in similar neurons, they do not co-localize. Hence, it is unlikely that NMR-1 and GLR-1 directly interact to produce long-term olfactory context conditioning. However, GLR-4 and GLR-5 are co-expressed in neurons expressing NMR-1 (Brockie et al., 2001b), in addition, studies in rodents show that, depending on the region of brain and training procedure, various subunits of AMPA glutamate receptors are dependent on NMDA mediated long-term plasticity (Toyoda et al., 2007; Han et al., 2009). Hence, testing GLR-4, GLR-5 and their associated proteins may elucidate a mechanism involving NMR-1 that underlies long-term olfactory context conditioning for mechanosensory habituation.

Vertebrate studies have established the importance of NMDA glutamate receptors (NMR-1) in associative learning because of its role as a coincidence detector and regulator of calcium influx (Yashiro and Philpot, 2008); because NMR-1 plays a role in context conditioning and CREB is regulated by calcium influx, the molecular pathway underlying memory between NMR-1 and CREB may also be conserved in C. elegans. One potential down stream target from NMR-1 and up stream from CREB in long-term olfactory context conditioning is cAMP dependent protein kinase (PKA or KIN-2); in the honeybee, over-expressing protein kinase activity enhances long-term associative memory (Muller, 2000). The \textit{kin-2(ce179)} allele, results in a holoenzyme that is hypersensitive to cAMP levels for PKA activation (Charlie et al., 2006), thus, if \textit{kin-2(ce179)} worms with a hyper-active PKA are tested in olfactory context conditioning, enhanced memory could be observed if PKA is in the NMR-1/CREB pathway in the
worm. PKA is also involved in the gene expression of chemosensory receptors (O'Halloran et al., 2009), hence another mechanism at the chemosensory neuron level may regulate long-term olfactory context conditioning.

Another possible mechanism for long-term olfactory context conditioning as discussed in Chapter 2 is changes in neuronal intrinsic excitability. These are non-synaptic (localized or neuron-wide) changes in cell membrane excitability mediated by voltage-gated ion channels in extra-synaptic areas; changes in neuronal intrinsic excitability have been observed in numerous long-term non-associative and associative learning paradigms in vertebrates and invertebrates, such as *Aplysia* and *Hermissenda* (Zhang and Linden, 2003). Since electrophysiological preparations have been successfully developed to study the AVA interneuron (a command interneuron in the context conditioning circuit; (Brockie et al., 2001b), comparing neuronal excitability between worms that received training for long-term habituation (non-associative), training for long-term olfactory context conditioning (associative learning) or no training, untrained naïve may help to determine whether intrinsic excitability of AVA plays a role in learning and memory in *C. elegans*.

GLR-1’s role in long-term olfactory context conditioning is consistent with work showing that GLR-1 is critical for long-term memory for mechanosensory habituation (Rose et al., 2003). In addition, Rose et al. (2003) showed that there was a strong correlation between the decrease in GLR-1::GFP and the expression of long-term memory for habituation. It will be important to determine whether this decrease in GLR-1::GFP is also seen in long-term olfactory context conditioning for habituation. If it is not, it suggests that long-term memory for context conditioning for habituation is
mediated by a completely different mechanism than long-term memory for habituation. If this is so, one possibility is that long-term memory for context conditioning is mediated by presynaptic changes; these have been extensively studied (Malenka and Bear, 2004). It is also possible to look for changes in the presynaptic marker synaptobrevin (SNB-1), a protein involved in synaptic vesicle regulation in the chemosensory neurons and the interneurons, to see if the associative context cue recruits additional processes in long-term memory for habituation. There was no SNB-1::GFP changes in the mechanosensory neurons after training for long-term memory for habituation at a 60s ISI using the presynaptic vesicular marker pmec-7::SNB-1::GFP (Rose et al., 2003).

Long-term olfactory context conditioning can be modified to study memory reconsolidation. Memory reconsolidation has been demonstrated in long-term memory for tap habituation in which GLR-1::GFP decreases, induced by consolidation were later reversed by reconsolidation blockade in C. elegans; this was the first time a mechanism for reconsolidation had been observed in any animal. Recently, Lukowiak et al. (2007) showed that snails were susceptible to memory infidelity of contextual cues, this has not been studied in C. elegans. Memory infidelity could be studied in C. elegans with olfactory context conditioning by using different volatile contextual cues during training and memory reactivation sessions; this may elucidate molecular mechanisms underlying memory infidelity (e.g changes in GLR-1::GFP expression). It will be interesting to see if presenting the context cue without the taps will reactivate memory in C. elegans; this type of memory reactivation has been demonstrated in the crab and was shown to be protein synthesis-dependent and mediated by NMDA-type glutamate receptors (Pedreira et al., 2002).
These results offer an opportunity to study the mechanisms underlying short- and long-term associative learning in *C. elegans*. A neural circuit analysis of short- and long-term memory will elucidate how a short-term memory recruits neurons additional processes to form longer-term memories. To see whether additional neurons are required for expression of long-term associative learning, testing a worm with NMR-1 only expressed in the RIM interneurons (*Ptdc-1::NMR-1* worms) will indicate whether one neuron is sufficient to rescue long-term olfactory context conditioning as seen in Chapter 2 for short-term context conditioning.

The data demonstrates long-term olfactory context conditioning for mechanosensory habituation, a form of associative learning shown for the first time in *C. elegans*. With only 302 neurons, worms can show complex forms of learning, which suggests a high conservation of memory mechanisms across phylogeny. A high conservation of memory mechanisms and the development of novel behavioral assays allow this genetic model system to investigate how different training procedures can be enhanced by a contextual cue and how cues from different modalities are integrated to alter these behaviors.
CHAPTER 4

4.1 General discussion

This thesis describes both short- and long-term chemosensory context conditioning for mechanosensory habituation, a form of associative learning in *C. elegans*. I have dissociated between taste and smell in short-term context conditioning and modified the training procedure to show long-term olfactory context conditioning in which glutamate neurotransmission played a critical role for both short- and long-term context conditioning.

In the experiments reported here, the context cue was either sodium acetate or diacetyl. The behavioral effects of exposure to sodium acetate and diacetyl warrant consideration. One possibility is that sodium acetate or diacetyl are arousing to the worm in such a way that tap reversal responses or their habituation are affected; the data described here suggest that this not the case because the wild-type worm’s response magnitudes and habituation are similar when presented with 30 taps during training in either diacetyl/sodium acetate or on plain NGM agar. Another possibility is that sensory adaptation to taste or smell cue would have caused worms to stop detecting diacetyl or sodium acetate, hence, the context cues are only detected within the first set of taps and no longer detected by the end of the training session. This is unlikely to be the case because Rankin (2000) showed that an extinction trial did not produce context conditioning, in this experiment the context cue was presented during the 1h rest period between training and testing. If worms no longer detected the context taste cue because of sensory adaptation, worms would not be able to determine that the taste cue no longer predicted the taps to show extinction or a lack of context conditioning. Thus the best
explanation for the behavior observed in the experiments reported here is associative context conditioning.

The associative nature of short- and long-term habituation is not surprising in the worm since stimuli are not presented individually in nature, but presented within a rich context of cues. The observation that context can be associated with habituation and enhance memory is consistent with Wagner’s associative theory of habituation (Wagner, 1981; Maldonado et al., 1997). What was surprising is that adding a context cue with habituation training at a 10s ISI led to the expression of long-term memory not seen in the absence of the cue. Further investigations of the cellular mechanisms of this form of memory, and comparisons with the mechanisms of long-term memory for habituation will determine whether this is a novel form of memory.

The memory processes underlying short- and long-term context conditioning is not well understood. In my experiment, I examined only a single time-point for short-term context conditioning (1h) and a single time-point for long-term context conditioning (24h). It will be important to investigate how expression of memory is regulated at different time points after training. Kamin (1957) found that memory expression of an avoidance response in rats follows a U-shaped time course after training, the “Kamin effect”, immediately high after training but decreases to a minimum 1h later then increases back to a stable level after several days. This has been studied in many organisms from invertebrates to humans (Gerber and Menzel, 2000), and is thought to reflect the existence of two independent and additive memory systems, one that dominates immediately after training and one that needs more time to consolidate to express memory (Kamin, 1957). To elucidate the consolidation process in context
conditioning, testing for memory retention at several time points after training will allow us to identify genes and mechanisms that may be responsible for early and late expression of memory for context conditioning.

Given that there are only 302 neurons, five mechanosensory neurons and eleven interneurons identified in the tap withdrawal circuit in addition to primarily six olfactory and sixteen gustatory chemosensory neurons (Bargmann, 2006), this is one of the most feasible organisms to perform a neural circuit analyses of learning and memory. To determine how neural circuits detect and integrate chemosensory stimuli, future studies should investigate how activation of various chemosensory receptors affect context conditioning. Chemosensation for smell is better understood than taste in *C. elegans*, so I plan to focus on a pair of AWA smell-detecting neuron, one of three pairs of olfactory chemosensory neurons in the worm, two that mediate attractive odors (AWA and AWC) and one that mediates aversive odors (AVA; Wes and Bargmann, 2001). Troemel et al. (1997) showed that AWA neurons can detect diacetyl and pyrazine among other odors, in which the chemoreceptor (ODR-10) for diacetyl has been identified and characterized. I hypothesize that training in diacetyl and testing in pyrazine, or vice versa, will produce context conditioning because they are both detected by the same chemosensory neuron and will cause similar activation down stream. Conversely, testing and training with different odors that are detected by different chemosensory neurons (e.g. benzaldehyde, detected by AWC) will not produce context conditioning; this will provide circuit level evidence of stimulus generalization and discrimination. Further, it would also be interesting to see how the worm responds to a compound stimulus during context conditioning when both are detected by the same neuron, different neurons or by a
combination of taste and smell. These studies will provide unique neural circuit-level insight into how chemosensory stimuli are integrated to alter subsequent behavior.

By analyzing the neural circuitry beginning with the AWA chemosensory neurons in long-term olfactory context conditioning, it maybe possible to address some of the mechanisms underlying memory infidelity during reconsolidation like that described in *Lymnaea* (Lukowiak et al., 2007). Lukowiak et al. (2007) showed that the memory for a context cue can be changed after training when a new context cue is exposed during a memory reactivation session; the time window and protein synthesis-dependence of memory infidelity was identified. During memory infidelity in *Lymnaea*, it is possible that a form of context cue (carrot-flavored vs. plain water) generalization was occurring; although the taste cue itself was different and can normally be discriminated however, during experimental conditions, the rest of the context (hypoxic water, other taste and smell cues, temperature, etc…) in the training and testing beaker remained the same in both carrot and plain context cue conditions. The generalization and stimulus-similarity components of memory infidelity in human studies for false memory and suggestibility work in a similar fashion: only “false” memories that are similar and likely to happen within the context of the memory are most vulnerable to suggestion (Loftus and Davis, 2006). Similarly in *C. elegans*, I hypothesize that only odors that are activated by the same olfactory chemosensory neuron or odors that activate the other attractive chemosensory neuron (AWC) will produce memory infidelity.

Vertebrate studies have established the importance of N-methyl D-aspartic acid (NMDA) glutamate receptors in associative learning, specifically involved in memory-mechanisms such as long-term potentiation, long-term depression and metaplasticity
(Yashiro and Philpot, 2008). The function of NMDA receptors are highly conserved in that NMDA receptors are also important for learning and memory in a number of invertebrate models such as *Aplysia* (Roberts and Glanzman, 2003; Glanzman, 2008) and recently in *C. elegans* (Kano et al., 2008). There are two identified NMDA receptor subunit homologs in *C. elegans* is NMR-1 and NMR-2 (Brockie et al., 2001b). Although only NMR-1 was tested in this thesis, it is likely that *nmr*-2 worms produce the same deficits in context conditioning as *nmr*-1 worms since Brockie et al. (2001b) suggested that NMR-1 and NMR-2 form heteromeric NMDA receptors because both mutations produce the same phenotype in associative learning assays and electrophysiological responses in AVA interneurons. The role of NMDA receptors needs to be distinguished from NDMA’s role in mammals before identifying NMDA as part of a memory mechanism for associative learning in *C. elegans*; NMDA receptors are not the only glutamate receptors to flux calcium as seen in vertebrates (Yashiro and Philpot, 2008), but AMPA receptors also flux calcium in *C. elegans* (Strutz-Seebohm et al., 2003). Although the NMR-1 receptor subunit may be homologous to vertebrate NMDA receptors, their function as coincident detectors is still unclear in *C. elegans*, Brockie et al. (2001b) could not confirm a voltage-dependent magnesium ion block like the one discovered in vertebrates. My data support the hypothesis of a role of NMR-1 in chemosensory context conditioning for habituation and together with Kano et al.’s (2008) associative learning study, suggest the hypothesis that *nmr*-1 maybe critical for all associative learning in *C. elegans*. In addition, because NMR-1 is involved in context conditioning in the worm, it possible that a form of long-term potentiation or long-term depression is occurring in AVA interneurons. In order to determine the long-term
changes in the AVA interneurons, calcium current recordings (Guo et al., 2009), patch clamp recordings (Brockie et al., 2001b), and expression of different glutamate receptors need to be investigated.

*C. elegans* provides a unique opportunity to identify cells and genes involved in neural plasticity, long-term changes in neurotransmission, synaptic strength or neural circuitry. *C. elegans* offer a clonal population with a fairly determinant cell lineage and predictable neural circuits, which allows an exquisitively sensitive measure of changes in neural changes in response to environmental cues. For example, the addition of a single chemosensory context cue during training and testing facilitates retention of habituation in which different genes and mechanisms are recruited during the learning process. With only 302 neurons, using well-characterized mutants I was able to identify a single gene (*nmr-1*) expressed in a single pair of interneurons (RIM) that mediate mechanosensory habituation in presence of context cues. In the future, I would like to distinguish the specific mechanisms and site(s) of plasticity that associate a single context cue to modulate a non-associative habituation process from mechanisms that are recruited for only the non-associative process. Future studies can either focus in or out at this point. To focus in on identified genes and proposed mechanisms to identify a molecular pathway in context conditioning, or to focus out by performing a genetic screen to identify novel genes and mechanisms to uncover the numerous ways animals integrate environmental stimuli into the nervous system to produce behavior. This study has shown that *C. elegans* can show complex learning in both the short- and long-term and that the temporal contiguity of environmental stimuli can have facilitatory effects on memory.
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