BEHAVIORAL ANALYSIS OF MEMORY IN CAENORHABDITIS ELEGANS

by

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ABSTRACT

Memory has been the focus of much research for more than 100 years. The earliest documented memory experiments were published by Hermann Ebbinghaus in 1885. In the current studies I investigated parameters affecting memory for habituation in the nematode *Caenorhabditis elegans* and determined several cellular mechanisms that underlie the behavioral expression of memory. I found that long-term memory for habituation requires a distributed training protocol. I confirmed the generally accepted view that mechanisms of memory consolidation occur after each training trial by showing that protein synthesis inhibition delivered after every training trial effectively blocked long-term memory. Glutamate has been postulated to play an important cellular role in memory consolidation; the findings of these studies confirm that long-term memory for habituation in *C. elegans* does not occur if glutamate transmission is compromised. I found that GLR-1 receptor activation is necessary for formation of long-term memory. Further, using a GFP transgenic strain to measure GLR-1 expression, I observed a significant decrease in GLR-1 24 hours after distributed training thus providing a putative mechanism for memory. I examined the permanence of this glutamate dependent long-term memory for habituation by inhibiting protein synthesis during memory reconsolidation and found that this effectively erased the earlier consolidated memory. Finally, I examined memory at an earlier time point (12 hours instead of 24 hours) and found that 12-hour memory can occur in the absence of 24-hour long-term memory and that 12-hour memory does not rely on distributed training, protein synthesis or glutamate transmission. My results showed that both a massed training protocol and a distributed training protocol produce memory 12 hours following training. Memory 12 hours after
massed training is mediated by a different mechanism than memory 12 hours after
distributed training. For the purposes of this dissertation, memory types are considered
distinct when they appear to rely on separate genes and/or mechanisms. Thus in this
research, I have dissected apart at least three distinct forms of memory. Together these
studies further our understanding of conditions required for memory to occur, and the
mechanisms upon which memory relies.
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ABBREVIATIONS

ANOVA - analysis of variance
AMPA - alpha-aminoo-3-hydroxy-5-
methylisoxazole-4-propionate
CAMP - cyclic adenosine monophosphate
CREB - camp-responsive element binding-
protein
DNQX - 6,7-Dinitroquinoxaline-2,3-dione
EPSP - excitatory postsynaptic potential
Fig - figure
H - hours
Hz - Hertz
ISI - interstimulus interval
ITI - intertrial interval
GFP - green fluorescent protein
LTD - long-term depression
LTH - long-term habituation
LTP - long-term potentiation
m - minute
ml - millilitres
msec - milliseconds
mRNA - messenger RNA
NaOH - sodium hydroxide
NMDA - N-methyl-D-aspartate
PCR - polymerase chain reaction
PKA - protein kinase A
PLSD - possible least significant difference
r - receptor
RNA - ribonucleic acid
RT-PCR - real time polymerase chain reaction
SNB - synaptobrevin
s - seconds
ul - microlitres
um - microns
YFP - yellow fluorescent protein

* by convention with C. elegans gene names are italicized while protein products are all caps
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CHAPTER 1

GENERAL INTRODUCTION

Memory is the mental storage of information and experience. As such, memory can allow for recall of a previous event and can be used to direct future behavior upon the re-experience of that or a similar event. One determinant of how memory is encoded is the temporal features of the experience. An experience can be seen as an aggregation of stimuli, so at the most basic level, memory mechanisms are likely encoding the temporal patterning of the stimuli that comprise an experience. The experiments presented here using the nematode *Caenorhabditis elegans* examine behaviorally how timing of stimuli determines memory encoding. Further, using the genetic tools available in *C. elegans* I have begun to uncover the underlying cellular mechanisms responsible for memory.

1.1 OVERVIEW OF SPACED TRAINING IN LONG-TERM MEMORY RESEARCH

There is evidence that the temporal patterning of stimuli plays an integral role in memory encoding dating back to 1885 when Hermann Ebbinghaus reported that his own memory for nonsense syllables lasted longer when memorization occurred in trials spaced over time compared to when he tried to learn the list of syllables in a single trial (as reviewed in Bourtchouladze, 2002). Further, the cumulative amount of time required to produce memory in a spaced training protocol was less compared to the amount of time required if memorization took place in a single massed trial. Even though less total
time was required for memory with spaced training, the resultant memory lasted longer than memory following a massed trial. A few years after Ebbinghaus' discovery, Muller and Pilzecker (1900) also investigated memory for nonsense syllables and found that some period of time must pass after learning for long-term memory to occur (see Lechner et al., 1999 for more detailed review). They called this period of time after learning the consolidation period, thus when training is distributed over time, this allows for more periods of consolidation.

Several studies have investigated the conditions under which spaced training enhances memory formation. Greene (1989) proposed that training in trials distributed over time allows for more cues to be encoded with the memory, thus making later retrieval of that memory more likely. This view was supported by Kahana and Greene (1993) who found that memory for homogeneous words (words belonging to the same category, e.g., four-footed animal) was not improved when memorization trials were presented in a spaced manner. More recently, Toppino et al. (2002) replicated this experiment and increased the number of presentations per words from two as was used in the Kahana and Greene (1993) experiment to three repetitions of each homogeneous word per trial and did find an effect of spacing; students were able to recall more words when memorization occurred in a spaced fashion.

Recently, using mice Scharf et al. (2002) found that the spacing effect may be specific to certain types of memory. They investigated memory for cued fear-conditioning where a foot-shock is delivered to the mouse in either a particular environment (context-conditioning) or following a tone (cued-conditioning) and found that long-term memory for contextual fear conditioning benefited from spaced
conditioning, while long-term memory for cued conditioning did not. A possible explanation for this is that in the case of context conditioning, there are more stimuli to be associated with fear (in other words, there is more to learn) and therefore context conditioning requires more training trials.

This advantage of spaced training for formation of long-term memory has been shown in several invertebrate animal models. In the crab *Chasmagnathus*, Hermitte *et al.* 1999 found that crabs will eventually habituate to the repeated presentation of a threatening stimulus (an overhead shadow). Further, crabs show long-term habituation after fewer presentations if stimuli are delivered in a spaced fashion. In the fruit fly *Drosophila*, long-term memory for an odor-shock association is only produced when repeated pairings are delivered in a spaced protocol (Tully *et al.*., 1994). In the sea slug *Aplysia*, spaced training was found to be optimal for producing long-term memory for habituation (Carew *et al.*, 1972). From these examples it appears that spaced training initiates some basic fundamental process that distinctly enhances formation of memory.

An important feature of spaced training that can influence its efficacy for long-term memory formation is the rate at which training exposures are delivered. There are two important features here, one is interstimulus interval (ISI)/intertrial interval (ITI) and one is interblock interval. Researchers who investigate memory for non-associative learning generally refer to this period of time as the interstimulus interval (ISI), while researchers who examine memory for associations between stimuli often refer to this period between CS-US pairings as the intertrial interval (ITI). Regardless, it seems that depending on the task, there is an optimal period of time between presentations for encoding long-term memory. Fanselow and Tighe (1988) found using contextual fear
conditioning that an ITI of 60s between context-foot shock associations was optimal for producing memory compared to shorter ITI’s (3s or 16s). Interestingly, Barela (1999) found that optimal ITI had an inverted-U function for memory for contextual fear conditioning in rats in that animals trained at a very long ITI (900s) showed memory at the same level as animals trained at the short ITI (15s), while the optimal ITI (60s) was the same as reported by Fanselow and Tighe (1988). However, this effect did not carry-over to cued fear conditioning as it appeared the longer the ITI, the more the animals remembered (Barela, 1999). Thus in humans and rodents, it appears that although it is generally accepted that spacing training over time produces better or more robust lasting memory, this effect seems to be somewhat task-specific and dependent on the number of repetitions per trial.

1.2 MECHANISMS OF LONG-TERM MEMORY

In 1949, Hebb postulated that when one neuron repeatedly and consistently activates another neuron, some neuronal or metabolic change results such that future activation of the second neuron by the first is facilitated. The first laboratory demonstration of Hebb's postulate came from long-term potentiation (LTP) in mammalian hippocampal neurons whereby brief bursts of high frequency electrical stimuli (~100 Hz) caused a long-lasting increase in the size of subsequent evoked responses (Bliss and Lomo, 1973) that lasts for hours in cell culture (e.g., Leutgeb et al., 2003) and days in vivo (e.g., in rats O’Boyle et al., 2004).

Over the years, research has uncovered several neuronal mechanisms that underlie this electrophysiological form of long-term synaptic plasticity. Various studies have
shown that LTP induction can depend on activation of either n-methyl-d-aspartate (NMDA) receptors (Debray et al., 1997). NMDA receptor dependent LTP is typically measured in the CA1 region of the hippocampus and relies on both the binding of ligand to the NMDA receptor and depolarization of the postsynaptic CA1 neurons to release the Mg\(^{2+}\) blockade from the Ca\(^{2+}\) channel of the NMDA receptor thus allowing an influx of Ca\(^{2+}\) into the postsynaptic neuron. Because these two events must take place in order for LTP induction to occur, this form of LTP has been termed associative. There is a second, nonassociative form of hippocampal LTP (found in the mossy fiber pathway) that relies on a single high-frequency burst. This single burst results in an increase in presynaptic calcium which eventually serves to increase transmitter release from the presynaptic neuron (glutamate; for overview see Squire and Kandel, 2002).

For NMDA dependent LTP, it appears that NMDA receptor activation may be crucial for LTP induction while non-NMDA-type glutamate receptors are involved in the maintenance of LTP. Recent evidence suggests that NMDA receptor activation of voltage-gated ion channels leads to the insertion of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-type glutamate receptors onto the post-synaptic membrane and that this process is required for LTP formation in the CA1 hippocampal slice. It is proposed that the increased density of AMPA receptors on the cell membrane provides sustained depolarization that may be necessary to initiate activation of gene transcription proteins (Lu et al., 2001). Further, activation of both glutamate receptor (AMPA and NMDA) sub-types have been implicated in the activation of adenylate cyclase and thus cyclic-adenosine-monophosphate (cAMP) formation, an enzyme known to activate cAMP-response-element-binding (CREB) protein which in turn initiates gene
transcription and protein synthesis. This CREB-dependent gene transcription can serve many functions including signalling the transcription of other genes (i.e., late-onset genes; Alberini et al., 1994).

When a new long-term memory is formed, it has been theorized that some growth process takes place to enhance signalling between the communicating neurons (Hebb, 1949). The new proteins produced following CREB activation are thought to be moved to the activated synapse and result in dendritic outgrowths, the accumulation of cell-adhesion molecules in the synapse (see Squire and Kandel, 2002). LTP induction has been found to result in CREB phosphorylation supporting the notion that gene transcription results from LTP (Davis, 2000). Behavioral studies employing the use of mouse genetic mutants has found that long-term memory is compromised in CREB mutant mice (Bourtchuladze et al., 1994), however this memory deficit can be rescued if spaced training trials are administered (Kogan et al., 1996). This latter finding suggests that CREB accumulation over time is enough to compensate for the decreased CREB activation seen with the CREB mutation and therefore long-term memory is restored. This also highlights the necessity of CREB activation and accumulation for long-term memory. It is likely that activity-dependent cellular changes thought to underlie memory rely on the activation of the CREB transcription factor.

What is difficult to ascertain from mammalian studies is whether hippocampal LTP induction truly underlies memory as it can only be correlated with hippocampal-dependent memory tasks, i.e., maze-tasks, spatial learning, etc. There is only a small amount of direct evidence that supports the hypothesis that LTP itself is the process by which memory is transformed into a permanent state. This drawback is in part due to the
greater neuronal number and degree of interconnections within and between brain structures of mammalian systems in addition to the greater complexity of the learning tasks investigated. This greater intricacy may be considered to be more relevant to memory as it occurs in humans, however, the precision with which one can study the neuronal mechanisms that underlie memory is lost within the noise and variability of a complicated system. Further, in a mammalian model system, memory for events is not necessarily localized to one region such as the hippocampus, because the various modalities used and integration of information required to perform laboratory tasks likely rely on neural networks that connect several brain regions (Schacter, 1996). Therefore, the view of one area being responsible for memory is somewhat limited. However, it is also virtually impossible for neurobiologists to understand intracellular mechanisms of memory while simultaneously adopting a whole-systems approach in a nervous system composed of billions of interconnecting cells.

Elucidating a specific neuronal mechanism responsible for memory required investigating at the level of the neuron. The work of Eric Kandel using the sea slug *Aplysia* has pioneered understanding synaptic signalling and the intraneuronal mechanisms presumed to underlie memory (see Squire and Kandel, 2002). This model system has simple reflexes (e.g., gill or siphon withdrawal reflex) that rely on relatively basic neuronal circuits found to be capable of supporting memory for non-associative learning paradigms including sensitization and habituation. A simple sensory-to-motor neuronal connection was thought to mediate these reflexes therefore researchers reconstructed this neuronal circuit in cell-culture allowing for physiological investigation of memory processes.
To induce long-term sensitization behaviourally requires distributed exposures to tail shock resulting in larger excitatory postsynaptic potentials (EPSP's) in the motor neuron and a corresponding increase in a defensive withdrawal behavior (Castellucci et al., 1986). This cellular analog of long-term facilitation can also be acquired if 4 distinct pulses of serotonin are applied every 20 minutes to the sensory neuron in cell-culture (Glanzman et al., 1989). The mechanisms underlying this long-term facilitation of response have been investigated extensively and it has been found that long-term memory for sensitization results in the increase in gene transcription factors such as CREB (Kaang et al., 1993) and the immediate early gene C/EBP (Alberini et al., 1994). As well, Bailey and Chen (1983; 1988) found a greater number of axonal varicosities, an increase in presynaptic active zones and a greater density of vesicles in those active zones resulting from long-term sensitization. Taken together, these experiments suggest that the mechanisms for long-term memory of a facilitated response occurs presynaptically and results in morphological, thus long-lasting, changes in the structure of the neurons that comprise the synapse.

Some aspects of the data supporting the presynaptic locus of memory in Aplysia have come into question as of late. First, mechanisms underlying long-term memory in Aplysia were hypothesized to occur in the presynaptic neurons exclusively, however, Glanzman et al. (1990) found no presynaptic morphological changes following long-term sensitization when presynaptic sensory neurons were cultured in the absence of corresponding postsynaptic motor neurons suggesting that the postsynaptic motor neuron has a role in long-term memory. As well, the neural circuitry of Aplysia is not necessarily as simple as once proposed. For instance, it has been found that the neuronal circuitry
that underlies the siphon withdrawal reflex is not monosynaptic but actually polysynaptic which may have implications as to how the animal responds to a stimulus being applied to the siphon (Trudeau et al., 1992). As well, optical measurements of the abdominal ganglion during gill-withdrawal following stimulation shows that over 200 neurons become activated in response to the stimulus (Falk et al., 1993). More importantly, the neurons of the abdominal ganglion are not uniformly activated during habituation with some neurons activating while others show no change or depressed activity. These additional connections may serve to modulate the sensory-to-motor connection in ways that were not controlled for during the initial behavioral and cell-culture experiments in Aplysia. Finally, Murphy and Glanzman (1996) reported that when the Ca\(^{2+}\) chelator BAPTA is infused into the postsynaptic motor neuron in culture, this blocks the cellular analog of classical conditioning (LTP).

Thus from these data it is clear that several biological processes are effected by repeated experience. Long-term memory in every model discussed here requires that training be distributed over time. As well, it is clear that a molecular cascade is initiated by repeated training that produces an increase in cyclic-AMP response element binding protein (CREB) and thus results in gene activation. Further, the gene products resulting from this process are likely responsible for consolidation of memory. Taken together, these data demonstrate how examining memory and plasticity in model systems has helped uncover mechanisms of memory.

1.3 Long-term Memory for Habituation
Habituation is defined as a decrease in response frequency or magnitude following repeated presentation of a stimulus (Groves and Thompson, 1970). This decrease in responding cannot be attributed to sensory adaptation or motor fatigue, thus habituation is characterized as a learning process. The specific mechanism underlying long-term memory for habituation (also called long-term habituation or LTH) has been more elusive. Under certain conditions, this decreased level of responding can still be seen hours (Beck and Rankin, 1997), days (Rose and Rankin, 2002) and weeks (Carew, et al., 1972) after the original repeated stimulus exposure. Habituation may be the simplest form of learning as it has been demonstrated even in single-celled protozoa (Wood, 1988).

The first report of long-term memory for habituation in Aplysia measured the siphon-withdrawal in response to an 800 msec jet of seawater. Carew et al. (1972) found that 40 presentations of the water jet delivered one every 30 seconds, in a single conditioning trial was not as effective at producing long-term memory as 40 trials divided into 10 trials per day (training trials were separated by ~24 hours). At testing (24 hours after the last training trial), animals initially showed recovery of the siphon withdrawal response to the water jet stimulus, but response decrement occurred faster in previously trained animals compared to control animals.

In a follow-up paper, Carew and Kandel (1973) found that 90 minute rest periods between training blocks were sufficient for producing reliable long-term memory. Further, electrophysiological recordings of EPSPs from the motor neurons responsible for the siphon-withdrawal response also showed a decreased potential when the corresponding sensory neuron was electrically stimulated at a rate matched to the
behavioral experiment (4 blocks of 10 stimuli delivered at a 30s interstimulus interval every 90 minutes). Interestingly, Carew and Kandel (1973) found a decrease in the EPSP of the motor neuron that matched closely to the behavioral decrement in siphon withdrawal suggesting that long-term memory for habituation was somehow encoded as a decreased probability of depolarization of the postsynaptic neuron. From this, experiments attempted to determine the cellular mechanism responsible for this decrease in the postsynaptic potential.

Although the mechanism by which memory for habituation operates is not yet fully understood, studies using electrophysiology and imaging have elucidated some of the likely processes responsible. By measuring EPSP probability Castellucci et al. (1978) determined that there was a significant decrease in detectable synaptic connections one day after long-term habituation training compared to untrained controls. With electron microscopy Bailey and Chen (1983) found a significant decrease in presynaptic structural elements associated with long-term memory for habituation including decreased number of varicosities, active zones and vesicles. From this it was proposed that long-term decreases in the behavioral response are the result of some presynaptic mechanism that serves to alter the morphology of the sensory neuron with long-term habituation resulting in a pruning of synaptic connections. Although long-term habituation has been found to act in a site-specific manner (Stopfer et al., 1996), at this time it remains unclear what intraneuronal machinery is responsible for this persistent decrease in response. Because distributed training trials are required for the induction of long-term habituation, it may be that similar processes that underlie long-term facilitation would also participate in long-term depression of responding.
One neural phenomenon that could be the mechanism for long-term memory for habituation is long-term depression (LTD). LTD has been observed in mammalian nervous systems and consists of a lasting decrease in the strength of a synaptic connection following low frequency stimulation. It could therefore be thought of as the opposite of LTP in that LTD produces a persistent and stable decrease in synaptic efficacy. LTD is measured at the same hippocampal sites as LTP. Further, LTD has also been shown to rely on NMDA receptor activation and Ca\(^{2+}\) influx into the postsynaptic neuron. However, unlike LTP that has been shown to rely on downstream cAMP-dependent kinases (e.g., protein kinase A; PKA), LTD activates a protein phosphatase cascade that results in the characteristic decrease in synaptic efficacy following LTD induction (reviewed in Malenka and Bear, 2004).

The similarities between habituation and LTD suggest LTD could be the cellular correlate for long-term habituation: habituation is a sustained decrease in responding to stimuli following repeated exposure while LTD induction requires repetition of low-frequency stimulation (~1 Hz) to the presynaptic neuron that results in a sustained decrease in EPSP's (depotentiation) in the postsynaptic neuron. This decrease in potential has been reported to last for hours in cell culture and for days in an acute in vivo preparation. Recently, Ezzedine et al. (2003) found that lasting memory for habituation relied on protein phosphatase, a protein also found to be necessary for LTD induction. In addition to relying on NMDA-type glutamate receptor activation, LTD can sometimes require AMPA-type glutamate receptor activation and results in an endocytosis of AMPA-type glutamate receptors (Carroll et al., 1999). This decrease in AMPA receptors at the synapse is hypothesized to mediate the lasting decreased potential measured from
the postsynaptic neuron, the opposite of what is proposed to occur with the increased potential seen with LTP. Because a decrease in excitatory receptors is seen with LTD, it follows that a similar decrease in excitatory receptors may underlie the sustained decrease in responding seen with long-term memory for habituation.

1.4 CAENORHABDITIS ELEGANS AS A MODEL TO STUDY MEMORY

One model system that has recently been studied in an attempt to investigate the characteristics and mechanisms of long-term memory for habituation is *Caenorhabditis elegans* (Beck & Rankin, 1997). *C. elegans* measures 1 mm at adulthood and is easily maintained in the laboratory: they live on agar filled petri dishes and survive on *E. coli* as their food substance (Brenner, 1974). The nervous system of this nematode is comprised of 302 neurons and these neurons have been anatomically mapped, therefore their cell lineage is understood, the connectivity between neurons is known (White et al., 1986; Chalfie, 1985) and their functional roles identified. In addition, the *C. elegans* genome has been completely mapped and sequenced and due to its hermaphroditic mode of reproduction, mutant strains are easily maintained.

*C. elegans* has been shown to exhibit a reversal response following a tap stimulus being applied to the side of its petri dish (see figure 1). This response has been reported to show habituation upon repeated tap presentation, as well as spontaneous recovery from habituation and dishabituation (Rankin et al., 1990). Laser-ablation studies have identified the neuronal circuit that underlies the tap-withdrawal response (see figure 2; Wicks and Rankin, 1995) and determined that the larger tap withdrawal circuit is comprised of two sub-circuits, one that produces a forward response while the other
Figure 1. Apparatus to Produce the Tap Stimulus. Worms swim on agar-filled Petri plates that are placed under a microscope in a holder attached to a micromanipulator. A copper rod attached to an electromagnetic relay taps the side of the Petri dish when a pulse is sent from the Grass 288 stimulator. A digital camera is mounted to the microscope that allows for viewing of the worm and recording of the response. Responses are scored onto acetate sheets at a later time, scanned into a computer file and reversal magnitude is measured in pixels.

produces reversals. Because a tap stimulus produces mechanosensory stimulation across the entire plate, both the reversal response circuit mediated by touch cells in the head and the forward response circuit mediated by touch cells in the tail are co-activated during a tap. Electrical gap junction connections from the mechanosensory neurons onto the interneurons mediate the tap-withdrawal response. However, mechanosensory neurons also make chemical connections onto the interneurons of the opposing circuit (i.e., touch cells in the head make chemical connections onto the interneurons responsible for forward locomotion Chalfie et al., 1985). Wicks and Rankin (1997) reported that these chemical synapses are the likely locus of plasticity suggesting that it is the synapses
Figure 2. Tap Withdrawal Response Neural Circuit. Rectangles represent the mechanosensory neurons, circles represent the interneurons that connect onto the triangles representing the motor neuron pools. Dotted lines represent gap junctions (electrical connections) while solid lines represent chemical connections. Darker neurons (PLM, AVB and PVC) mediate forward locomotion while lighter-shaded neurons (ALM, AVM< AVA and AVD) mediate reversals (swimming backwards). It has been proposed that the tap stimulus activates both circuits, however, the reversal circuit is usually stronger resulting in a reversal response to tap 90% of the time. (adapted from Wicks and Rankin, 1995).

between the mechanosensory and interneurons that undergo changes due to learning. This information allowed me to direct my investigations towards genes that are specifically expressed in the neurons of this identified neural circuit.

Previous experiments have examined long-term memory in C. elegans and have shown that distributed training of response habituation to a mechanosensory stimulus in C. elegans resulted in retention of the habituated response 24 hours later while massed training did not (Beck and Rankin, 1997). In addition, only worms that received distributed training at long ISIs (60s) demonstrated long-term retention of the habituation learning; worms that received either massed training at a short (10s) or long ISI (60s) or distributed habituation training at a short ISI (10s) showed no retention 24 hours later (Beck and Rankin, 1997). As well, Beck and Rankin (1995) demonstrated that if heat
shock was administered during the rest period between training blocks 24-hour retention for habituation training was abolished. Heat shock results in the production of heat shock proteins that function to prevent denaturing of proteins during periods of cellular stress (Lindquist, 1986) resulting in the interruption of protein synthesis. Thus, long-term memory for habituation was shown to rely on protein synthesis that occurred after each training trial. Together these data confirmed that the long-term memory for habituation in C. elegans is similar to what has been found in other organisms and provides the basis for the experiments in this dissertation.

The chemical connections between the mechanosensory neurons and the interneurons in the tap-withdrawal circuit have been reported to be glutamatergic (Avery, 1993; Hart et al., 1995; Maricq et al., 1995). For example, the eat-4 gene (codes for a glutamate vesicular transporter) is expressed in the mechanosensory neurons and mutation of the eat-4 gene affects availability of glutamate (Avery, 1993). Because of evidence from other studies of memory and/or neural plasticity regarding the importance of glutamate transmission (mentioned above), investigating the role of genes involved in glutamate transmission was an obvious direction for me to pursue in this research.

1.5 RELATIVE PERMANENCE OF LONG-TERM MEMORY

The stability of long-term or consolidated memory has received much interest (see Dudai and Eisenberg, 2004a for review), in that several studies have demonstrated a seeming erasure of a previously established memory. Originally, Misanin et al. (1968) found with human patients that electroconvulsive shock after training did not affect memory performance, however, if shock was delivered after a training cue, later memory
performance was impaired. More recently, Nader et al. (2000) found using an associative fear conditioning task with rats, where memory for a tone-footshock pairing was measured, that if the protein synthesis inhibitor, anisomycin, was administered after memory reactivation by later presentation of the tone alone, rats showed no memory of the tone-footshock association at a later test time. Inhibition of memory was specific to anisomycin being administered at the time of the tone-alone, if anisomycin was delivered in the absence of the tone, this did not affect memory. Nader et al. (2000) postulated that the tone was serving as a cue to the earlier tone-footshock association and that this recall resulted in the reactivation of that specific memory trace. Further, they postulated that memories that are reactivated are also made malleable or labile again and require reconsolidation of the memory trace back to a more permanent state. Thus, when protein synthesis was inhibited after memory reactivation, this inhibited subsequent memory reconsolidation and ultimately led to the abolition of the previously consolidated memory. The conclusions drawn from this study include: reactivation of memory returns that memory to a labile state, both the initial consolidation of a memory and later reconsolidation rely on protein synthesis dependent processes, inhibition of reconsolidation abolishes the previously established memory.

This finding inspired research investigating this phenomenon into reconsolidation: the theory that retrieval of a previously consolidated memory returns all or part of the memory to a modifiable or labile state. Many researchers have since investigated this phenomenon using memory for various associative learning tasks in several animal models (Milekic and Alberini, 2002; Anokhin, Tiunova and Rose, 2002; Sangha et al., 2003a; Cammarota et al., 2004; Wang et al., 2005). Since the experiments in the first part
of this dissertation allowed me to characterize the conditions under which long-term memory can be produced in *C. elegans*, as well as some of the molecular mechanisms that likely underlie this memory, it was of interest to determine if this same long-term memory would also be vulnerable to inhibition of memory reconsolidation.

### 1.6 MEMORY EXISTS IN PHASES

What has become obvious from experiments in mammals and *Aplysia* is that long-term memory formation requires a period of consolidation whereby short-term memories are transformed into a more permanent form for long-term memory. LTP, the proposed molecular correlate of lasting memory discussed earlier, has been shown to have at least two separate phases; early-LTP (E-LTP) produced by a single tetanus of high frequency stimulation that lasts 1-3 hours and a late-LTP (L-LTP) produced by four tetanus trains given at 10 minute intervals, that lasts more than 24 hours (Nicoll *et al.*, 1988). Similar to what has been demonstrated with long-term memory, L-LTP relies on gene transcription and protein synthesis, however, E-LTP does not.

However, there is considerable research on memory that suggests that it cannot be divided down the middle into two distinct stages: short- and long-term memory. There appear to be a number of distinct phases through which memory must either pass through to become long-term memory, or that exist in parallel with long-term memory. This has been studied in a number of different systems (e.g., rat (Rosenzweig *et al.*, 1993), the honeybee *Apis mellifora* (Hammer and Menzel, 1995), the snail *Lymnaea stagnalis* (Sangha *et al.*, 2003a), etc.). Here I will focus on three of these: *Drosophila, Aplysia* and the chick. Memory for odor avoidance conditioning in *Drosophila* (where a particular
odor is paired with shock and later avoidance to the previously paired odor is the measure of memory) occurs in two distinct long-term (>24 hours) forms: anesthesia-resistant memory (ARM) and long-term memory ([Tully et al. 1994]). ARM was found to result from massed training, is not effected by cold shock nor pharmacological inhibition of protein synthesis, and lasts up to 4 days whereas long-term memory results from distributed or spaced training, is abolished by protein synthesis inhibition and persists for at least 7 days (for review see [DeZazzo et al., 1995]). As well, ARM is abolished in flies carrying the radish (encodes a phospholipase-A2; Chiang et al., 2004) mutation. While long-term memory in Drosophila appears to require the CREB gene; mutations that upregulate the level of activator CREB enhances long-term memory ([Yin et al., 1995]) and expression of a dominant negative CREB transgene abolishes long-term memory ([Yin et al., 1994]). ARM remained intact in flies that expressed the dominant negative CREB transgene. These data support the hypothesis that long-term memory depends on CREB activation and gene transcription while ARM does not require CREB, but does require radish. More recently, Isabel et al. (2004) reported that ARM and LTM may actually exist as exclusive, competing forms of memory. Together, these data show the genetic differentiation of two lasting memory phases and lend support to the notion that there are multiple mechanisms of memory.

Similarly, in Aplysia, long-term retention (>24 hours) for sensitization (an increased response to a weak stimulus when preceded by a noxious stimulus) requires a distributed or spaced training protocol ([Pinsker et al., 1973]) and relies on protein synthesis ([Castellucci et al., 1986]). However, Sutton et al. (2000) reported another lasting form of memory that is seen 90 minutes after training (intermediate-term memory) that
could be produced with either massed or distributed training. Unlike what has been reported in *Drosophila*, this intermediate-term memory phase declines completely before the onset of long-term memory, is protein synthesis dependent but does not require new RNA synthesis and relies on activation of protein kinase A (Sutton *et al.*, 2001).

Less is known in regard to long-term memory for habituation in *Aplysia*, however, it has been reported that lasting memory for habituation also relies on repeated presentation of the habituating stimulus presented in a spaced manner (4 blocks of training separated by 24 hours or 90 minutes; Carew *et al.*, 1972; 4 blocks of 20 habituating electrical pulses with each block separated by 90 minutes; Ezzedine and Glanzman, 2003). To date, there has been no reported form of memory for habituation that equates to an intermediate memory.

The experiments on memory stages in *Drosophila* and *Aplysia* suggest that multiple memory mechanisms can be initiated in parallel; in contrast memory in chicks appears to be a serial process. The day-old chick has been used to examine long-term memory because it has the ability to form permanent memories relatively quickly. The avoidance paradigm employed for these studies consists of day-old chicks being presented with several tiny colored beads. Beads of a specific color are dipped in a bitter tasting substance chicks dislike (e.g., methylantranilate) that upon pecking display a typical disgust reaction of head shaking and beak wiping. If presented with a bead of the same color at a later time point, chicks will avoid pecking those beads. What has been found in this system is that long-term memory for this single-trial avoidance task requires serial progression from short-term memory (<10 minutes), through intermediate-memory (15-30 minutes) to be consolidated into long-term memory (1 hour; Gibbs *et al.*, 1979).
These phases of memory occur in a serial fashion with dips in memory 15 and 55 minutes post-training during which memory is thought to be moving between phases (going from short- to intermediate-term or intermediate- to long-term memory, respectively). This is in contrast to the parallel ARM and LTM memory processes found in *Drosophila*.

What is interesting to note is that the memory seen following odor avoidance conditioning in the fruit fly required repeated trials, and that the type of memory seen following training depended on the time parameters of the training protocol (massed vs. distributed training). The chick example looked at memory following one-trial conditioning, therefore, it may follow that memory for repeated stimuli depend on timing and activate several forms of memory simultaneously while one-trial learning may recruit a single memory process that has a more continuous nature.

Thus it remains to be determined whether memory is a graded progressive series of processes or whether there are several different memory processes that can be activated simultaneously. What is clear from this data is that in every system in which memory has been dissected on the basis of mechanism there are multiple mechanisms of memory, not just one for short-term and one for long-term.

1.7 OVERVIEW OF OBJECTIVES

Based on the observations of Beck and Rankin (1995; 1997) regarding the ability of *C. elegans* to demonstrate long-term memory I carried out a research program to address four objectives to further the understanding of properties of memory; 1) develop a more efficient training protocol that reliably produces long-term memory in *C. elegans*
and confirm that long-term memory relies on protein synthesis-dependent consolidation,
2) determine the role of glutamate in long-term memory, 3) determine whether different
forms of memory could be measured at shorter retention intervals by manipulating
training parameters, and 4) determine whether the long-term memory seen in C. elegans
can be modified once consolidated.

The research conducted for this dissertation is organized into four sections, each
describing experiments that address one of the four objectives of the research:

Objective 1) **To establish a more efficient training protocol to produce long-term
memory for habituation.** Previously, Beck and Rankin (1997) developed a training
protocol to examine long-term memory for habituation in C. elegans whereby each worm
was trained and tested alone on an individual plate and the worm's response in the test
phase was directly compared to its own response during the training phase. The goal of
establishing a new training protocol that produced long-term memory was that the
previous within-subjects design was very time-consuming, taking more than 7 hours per
worm. In the first set of experiments in this dissertation I modified the original memory
training protocol established by Beck and Rankin (1995). This original protocol was a
within-subjects design where responses made during training and testing were recorded
and measured. Responses between training and testing were compared for each individual
worm. I modified this long-term memory training protocol to a between-subjects design
where responses between groups (trained vs. untrained) were compared. For this new
experimental design, worms were trained together on a single petri dish, transferred one
hour after the last training block onto individual plates and then tested alone thus
increasing the throughput dramatically. Because we were unable to record responses for a whole dish of worms, the training phase went unrecorded. Instead, the responses of trained worms were compared to test phase responses of an untrained control group that received a single tap on training day. Once we confirmed that long-term memory could be produced with this new, faster protocol, we set to determine if this memory was comprised of the same characteristics as previously reported with the original memory training protocol (Beck and Rankin, 1995; 1997); namely, required distributed training, stimuli delivered at a long interstimulus interval (60s) and relied on protein synthesis.

Objective 2) The role of glutamate in long-term memory in *C. elegans*. Glutamate transmission and receptor activation have been implicated in both long-term memory for behavioral tasks and long-term synaptic plasticity (see above). In *C. elegans*, the neural circuit that has been hypothesized to mediate the tap-withdrawal response is glutamatergic and therefore it is of interest to both worm and memory researchers alike to understand the role of glutamate itself and glutamate receptor activation in memory formation. To this end, I investigated whether the new training protocol was sufficient for examining long-term memory in worm strains that carry genetic mutations for specific genes. For this experiment, I studied the role of glutamate transmission in long-term memory by subjecting worms of the *eat-4* mutant strain (limits the availability of glutamate) to both the within-subjects training protocol and the newly established between-subjects training protocol. Once we confirmed that the new training protocol could be used for measuring long-term memory in mutant strains as effectively as the
original training protocol, we employed the new between-subjects training protocol exclusively.

To further our understanding of the role of glutamate in long-term memory, we also investigated the role of the glr-1 gene (homologous to the mammalian AMPA receptor) in long-term memory. My results from the glr-1 mutant strain were confirmed with a pharmacological experiment whereby worms were trained in the presence of 6,7-Dinitroquinoxaline-2,3-dione (DNQX; a non-NMDA-type glutamate receptor blocker), and tested on non-drug plates. From these experiments we were able to determine the importance of glutamate and glutamate receptor activation in long-term memory.

In this series of experiments I also made use of another genetic technique available in C. elegans; imaging transgenic strains that carry a green fluorescent protein (GFP) construct attached to a particular gene. This allows the examination of changes in the expression of a specific gene due to experience. I examined two transgenic strains, GLR-1::GFP serves as a postsynaptic marker of glr-1-containing glutamate receptors and pmec-7::SNB-1::GFP which serves as a presynaptic marker of vesicles. From these studies I have begun to piece together the mechanism at work for long-term memory.

Objective 3) **Examine the Permanence of Long-Term Memory.** Recent studies have demonstrated that if protein synthesis is inhibited following retrieval of a previously consolidated memory, this inhibits subsequent recall of the memory at a later time. The hypothesis for this phenomenon is that when memory is reactivated, it somehow returns to a labile state thus making it vulnerable to disruption (see Nader, 2000 for review). The results from my previous studies have established and characterized long-term memory
for habituation in *C. elegans* as a reliable memory process. I understand the training conditions required to produce this long-term memory (i.e., distributed training) and the manipulations that interrupt formation of this long-term memory (i.e., protein synthesis inhibition) are the same as has been reported for long-term memory for other tasks in other organisms.

With this knowledge, I set to determine whether this long-term memory is also sensitive to disruption by inhibition of protein synthesis following memory reactivation. To study this, I trained worms with a distributed habituation training protocol and then delivered a reminder session 24 hours after training. I administered heat shock immediately after this reminder session and then measured memory at 24 hours after reminder. To gain an understanding of the possible mechanism underlying this reconsolidation of memory, I also examined the effect of delivering reminder training in the presence of the non-NMDA-type glutamate receptor blocker, DNQX. Finally, I also imaged the GLR-1::GFP and *mec-7::SNB-1::GFP* transgenic strains at 24 hours after reminder to determine if memory modification could be correlated with changes in either *glr-1* or vesicles. I found that both the behavioral expression of memory and the mechanism thought to underlie memory were both disrupted by inhibition of protein synthesis after retrieval.

Objective 4) **Different Training Protocols Can Produce Retention at Shorter Intervals.** In investigating objectives 1 and 2, I demonstrated that worms show 24-hour retention for habituation if training is administered in distributed blocks but results in no long-term retention if training is massed in a single block (Beck and Rankin, 1997; Rose
et al., 2002). As well, long-term memory formation is attenuated if protein synthesis is blocked with heat shock during the first 40 minutes after training (Beck and Rankin, 1995; Rose et al., 2002). I next examined whether distributed or massed training would produce memory at a shorter retention interval (i.e., 12 hours). I manipulated the time parameters of the long-term memory training protocol (i.e., massed versus distributed training at a short ISI; 10s). Once memory at 12 hours was confirmed, I examined the role of protein synthesis in this putative memory phase. As well, imaging of the same GFP transgenic strains used in the second set of experiments, allowed me to determine that memory at this earlier time point did not result from the same cellular mechanisms as 24-hour long-term memory. Because glutamate was not important for 12-hour memory, I investigated the role of another group of C. elegans genes (flp, FMRFamide-like-peptides) in both 24-hour long-term memory and this earlier memory 12 hours after training. From these experiments I was able to uncover and characterize at least two genetically dissociable memory phases in C. elegans.
CHAPTER 2

Objective 1: To establish a more efficient training protocol to produce long-term memory for habituation.

2.1 INTRODUCTION

Remarkably little is known about the biological foundations of long-term habituation, thus one of the objectives of the current study is to develop *C. elegans* as a model system in which to investigate the cellular mechanisms of long-term memory for habituation training. In previous studies of long-term memory *C. elegans* were individually trained with three blocks of trains of taps (60s ISI) separated by one hour rest periods (taking about 4 hours per worm) and then tested 24 hours later with 20 trains of taps at a 60 s ISI (Beck et al., 1995; 1997). Although this procedure was successful in producing long-term habituation it was very time-consuming. In this first series of experiments I tested whether a new protocol, in which groups of worms were trained simultaneously with four blocks of 20 single taps followed by one-hour rest periods, would lead to long-term memory when worms were tested with 10 single taps at a 60 s ISI 24 hours later.

The other modification I made to the original training protocol was the stimulus used for habituation training. In Beck and Rankin (1995; 1997) the habituating stimulus was a train of taps whereby 6 taps were delivered in rapid succession to the side of the worm’s petri dish at a rate of 6 taps/second. The new *en masse* protocol uses a single tap rather than a train of taps. Although a single tap could be considered a milder stimulus
and therefore short-term habituation would be presumed to be enhanced (Groves and Thompson, 1970), it was not clear what affect this would have on long-term memory for habituation. Because changing the stimulus to a single tap allowed for faster video-scoring analysis I decided to test its efficacy for long-term habituation. Taken together, these two differences reduced the time it takes to conduct a single long-term memory experiment from several months to a few weeks.

In previous studies of long-term memory in *C. elegans*, Beck and Rankin (1995) found that inhibition of protein synthesis by heat shock effectively blocked memory. Heat shock results in a cellular stress response that produces heat shock proteins. These heat shock proteins function to prevent the denaturing of other cellular proteins by heat stress. The result of the production of heat shock proteins is the inhibition of the synthesis of any new proteins (Schlesinger et al., 1982; Duncan et al., 1984; Lindquist, 1986). Beck and Rankin (1995) showed that heat shock prior to training, or prior to testing had no effect on long-term memory for habituation indicating that neither the experience of heat shock alone, nor the presence of heat shock proteins was sufficient to disrupt memory. The current study sought to replicate the disruption of long-term memory for habituation seen by Beck and Rankin (1995; 1997) by delivering heat shock after each training block in the new procedure and examining memory 24 hours after training.

In other invertebrate systems cold shock has been used as a method for inducing retrograde amnesia (e.g., Tully et al., 1994; Sangha et al., 2003b), however relatively little is known in regard to the mechanism by which cold shock functions. When an organism experiences very low temperatures, cells begin to produce cold shock proteins. This process has been shown to be conserved from bacteria to man (Wolfe et al. 1992).
In terms of protein synthesis, cold-inactivated ribosomes first exclusively synthesize cold shock proteins (e.g. due to intrinsic properties of their mRNAs), which then alter ribosome conformation thus disabling protein synthesis (for review see Weber and Marahiel, 2002). Cold shock will be administered after each learning trial to determine if a comparable inhibition of memory occurs as has been reported in other systems.

To ensure I was producing the long-term memory comparable to that described by Beck and Rankin (1995; 1997) with this new training protocol, I investigated whether this memory had the same characteristics as memory seen following the original protocol. These included: requiring a distributed training protocol, habituating stimuli delivered at a long interstimulus interval (ISI; 60s) and protein synthesis dependence. The goal of this work was to establish a faster training protocol to conduct experiments with genetic mutant strains to uncover genes necessary for memory.

2.2 METHODS

Subjects

A total of 469 hermaphroditic *C. elegans* Bristol (N2) originally obtained from the *Caenorhabditis* Genetics Center were used in these studies. All worms were maintained on Nematode Growth Medium (NGM) agar seeded with *E. coli* (OP50) as described in Brenner (1974).

Behavioral Testing

Habituation training and testing took place under a stereomicroscope (Wild M3Z, Wild Leitz Canada) connected to a digital camera (Panasonic Digital 5100), VCR
(Panasonic AG1960) and monitor (NEC). In all recorded conditions a time-date generator (Panasonic WJ-810) superimposed the time-date on the video record in addition to a digital clock that counted to the hundredths of a second. In the new protocol the training was not recorded as worms were trained in groups which prevented me from scoring the responses of individual worms. Therefore the training phase occurred without the microscope stage illuminated. Testing occurred with the stage light on and was video-recorded. Habituation stimuli (taps) were administered via electrical stimulation from a Grass S88 stimulator resulting in a mechanical tapper exerting 1-2 N of force to the side of the petri plate on which worms were kept producing a single tap stimulus.

The new group-training distributed protocol consisted of an experimental group of 15-20 4-day old worms trained simultaneously on a single plate with 4 blocks of training. Each block consisted of 20 single tap stimuli given at a 60 s ISI with one-hour rest periods between blocks (see figure 3). Single taps were used in this protocol to shorten the time required to score the video-recorded responses; scoring the stronger “train of taps” stimulus required more time. A control group that included another 15-20 worms received only a single tap on training day. At least one hour after the last tap of training worms from both the trained and single-tap control were transferred on to labeled individual *E. coli* streaked plates. Worms from both groups were tested one at a time with 10 single taps given at a 60 s ISI: testing occurred 22-28 hours following training (median >24 hours).

If the original training protocol was employed then the training phase occurred with the microscope stage light on to allow for video-recording of responses. In the new protocol the training was not recorded therefore the training phase occurred without the
microscope stage illuminated. In the original procedure, memory was measured by comparing the responses to the 20 stimuli delivered on test day to the responses of the first 20 stimuli delivered on training day. The new group-training protocol does not allow for gathering of data during the training phase. Therefore, in this protocol, memory is measured as a comparison of average response magnitude to the 10 test single taps between the trained group and an untrained matched control group. Long-term memory for habituation training was measured as a significant difference using a between-groups comparison for the test responses for the experimental and control groups. With this protocol it took approximately 14 hours to train and test 20 experimental and 20 control worms.

Figure 3. Experimental Protocols for Measuring 24-hour Retention of Habituation. Top illustration shows massed training protocol while the bottom illustration shows the distributed training protocol. Testing consisted of 10 taps delivered at a 10s ISI to both trained and untrained worms. Responses during the test phase were compared between groups.
worms compared to approximately 175 hours to train and test 20 experimental and 20 controls worms with the original training protocol.

For massed training in the new protocol an experimental group of 15-20 worms received one block of 80 stimuli at a 60 s ISI on training day and a control group of 15-20 worms received only a single tap stimulus. At least one hour after the last tap of training worms from both the trained and single-tap control were transferred onto labeled individual plates. Twenty-four hours later both groups were tested one at a time with 10 single taps given at a 60 s ISI. The mean reversal magnitude across the 10 test single taps was compared between the two groups. Long-term memory for habituation training was measured as a significant difference between the trained and control groups on test day.

When I examined the ISI-dependence of this long-term memory, the training and testing procedures were identical except that the ISI between taps during both the training and the testing was 10 seconds. For the study examining the effect of a shorter interblock interval, the identical training parameters were employed except the rest periods were only 40 minutes instead of one hour.

**Scoring**

Video records of the training and testing phases (original protocol) or of the test phases of the trained and single-tap control group (new protocol) were scored using stop-frame video analysis onto acetate sheets. These tracings of reversal magnitude were then scanned into a computer and measured using NIH image software. Previous research reported that 90% of the time adult worms respond to a single tap or train of taps with reversals (Chiba and Rankin, 1990), therefore, because all experiments presented here
used adult worms for training and testing it was expected that worms would respond a majority of the time to a single tap or train of taps with a reversal. However, over the course of an experiment a worm occasionally accelerated forwards to tap. The neural circuit for accelerations forward is different from the neural circuit for reversals to tap, and habituates with different kinetics (Wicks and Rankin, 1995, 1996). Therefore, accelerations were considered qualitatively different from reversals and were scored as missing data points. Fewer than 10% of the data points fell into this category. If the worm paused in response to the tap/train or did not respond to the stimulus, then this was scored as zero.

Data Analyses

All data analyses were conducted using Statview 4.5. Reversal magnitude values were averaged across the test phase. The responses of experimental and control groups were compared using a two-tailed unpaired t-test. Where more than two groups were compared, an analysis of variance (ANOVA) was used to determine if any difference between the group means was statistically significant. Because it is the difference between each respective control and trained group that is of interest to this study, planned comparison t-tests (with a Bonferonni adjustment of the alpha level for multiple t-tests) were used when ANOVAs yielded a significant result. Responses are reported as percent control response whereby the responses of both the trained and untrained control groups are normalized to the mean of the untrained control group and multiplied by 100.

Heat Shock Treatment
In an experiment in which worms received heat shock treatment, both the trained group and the matched tap control group underwent heat shock. Heat shock was given by double-wrapping Petri plates with parafilm on the training day and submerging them in a 32°C bath for the first 40 minutes of each 1 hour rest period. During the last 20 minutes of the one-hour rest periods training plates were returned to room temperature. For comparison, the no-shock control group underwent the same procedure except that plates were submerged in a room temperature (21°C) bath.

2.3 RESULTS

Experiment 1A. The New Habituation Protocol Replicates the Findings of the Original Protocol

The first stage of establishing the new group-training protocol for studying long-term memory for habituation training in *C. elegans* was to replicate the findings of Beck and Rankin (1995, 1997). The protocol used in Beck and Rankin (1995, 1997) consisted of 3 blocks of distributed training using “trains of taps” as the habituation stimulus whereas the new protocol consists of an additional 4th block of training and employed single taps as the habituation stimulus. In the first experiment with the new protocol the effects of massed and distributed training were tested. There were 4 groups of wild-type worms tested: a distributed training group (4 blocks of 20 single taps at a 60 s ISI separated by 1 hour; n=27) and its matched control group (n=24) as well as a massed training group (1 block of 80 single taps at 60s ISI; n=22) with its tap control group (n=22). The results can be seen in Figure 4 in which both the raw test data and the mean response across testing are shown. In figure 4A showing the raw data for the distributed
training, all responses from trained worms were lower than the responses for the untrained control group, whereas there was great overlap between responses of trained

Figure 4. Distributed training but not massed training produces long-term memory for habituation. Line graphs illustrate the average reversal response magnitude, standardized over worm length, for each tap during the test phase for A) a group that received distributed training and its matched single-tap control group, and B) a group that received massed training and its single-tap matched control group. Differences between mean response magnitudes across testing phases between a trained group (white bars) and respective single-tap matched control group (black bars) were compared between groups that received habituation training distributed in blocks or given in a single massed training trial (C). Mean response magnitudes are expressed as percent control group response. ** = p < 0.01.
and untrained worms in the massed condition (figure 4B). An ANOVA showed a significant difference (F(3, 91) = 4.098, p≤0.005). Planned comparisons revealed that the mean response to the 10 test single taps by the group that received distributed training was significantly smaller than the mean response to the 10 test single taps for the matched tap control group (t(49) = 3.03, p≤0.01; Figure 4C). When the mean response to the test taps was compared for the massed trained group and its matched tap control group there was no significant difference in response magnitudes (t(42) = 0.52, p>0.10). These results replicate the findings of Beck and Rankin (1997); distributed training produced long-term memory for habituation, while massed training did not.

Beck and Rankin (1997) also found that worms that received distributed long-term habituation training at a short (10s) ISI did not show retention of the habituation training 24 hours later. The next experiment tested whether delivering stimuli at a 10 s ISI in the new group-training protocol would also fail to produce long-term memory. The procedure for this experiment was the same as the distributed training procedure described above, except that within blocks stimuli were delivered at a 10 s ISI rather than a 60 s ISI. ANOVA revealed no significant difference between trained and untrained worms on test day when a 10 s ISI was employed with the group-training protocol with either massed or distributed training (F(1, 79) = 1.098, p≤0.298; Figure 5A) when each was compared with its tap control group. Thus, this experiment replicated Beck and Rankin (1997) by showing that no long-term memory for habituation was produced with the group-training protocol when the stimuli were delivered at a 10 s ISI.

Beck and Rankin (1995) reported that long-term memory for distributed habituation training with the single worm protocol could be blocked if heat shock was
administered in the one hour interval between blocks of training. Heat shock stopped on-going protein synthesis and blocked new protein synthesis from occurring for a critical period of time after each training block (Schlesinger et al., 1982; Duncan et al., 1984; Lindquist, 1986). In this experiment the effects of heat shock on the new group-training protocol were tested. There were four groups in this experiment; the first received the normal distributed training protocol with no heat shock treatment (no shock group), the second was its tap control group. The third group received the distributed training

**Figure 5.** The new group-training protocol replicates previous findings that distributed training at a short ISI does not produce long-term memory for habituation training (A) and that heat shock given during the rest periods that separate the distributed training blocks eliminates long-term memory (B). A) The mean response magnitude for trained groups (white bars) that received habituation training at a 10s ISI using either a massed or distributed training protocol compared to their respective single-tap matched control group (black bars). Mean response magnitudes are expressed as percent control group response. B) Mean response magnitudes for groups that received distributed training with heat shock given in the first 40 minutes of each one-hour rest period (white bar) do not differ from mean response magnitudes of the matched control group that also received heat shock (black bar). A control condition where trained worms received distributed training and then underwent 21°C (room temperature) shock during the rest periods between training blocks (white bar) show significantly decreased mean response magnitudes compared to its matched control group (black bar). Mean response magnitudes are expressed as percent control group response. * = p < 0.05.
protocol, and was given heat shock treatment for 40 minutes following each of the four training blocks (heat-shock group). The fourth group was tap control for group 3, and received the same exposure to heat shock as the trained group, but only a single tap on day 1. An ANOVA was significant \( F(3, 84) = 3.297, p \leq 0.024 \). Planned comparisons revealed normal long-term memory in the no shock treatment training group \( t(50) = 2.278, p \leq 0.027 \) and, in the heat-shock group, heat shock blocked the long-term memory for habituation normally produced by the group-training protocol \( t(34) = 0.181, p > 0.10; \) Figure 5B).

Although in our previous experiments, we have always used heat shock to block ongoing protein synthesis, studies of memory in other species use cold shock (Tully et al., 1994; Sangha et al., 2003b). We next investigated whether cold shock could be used to block memory in *C. elegans*. Cold shock (0°C) was administered after distributed and after massed training and memory was examined 24-hours later. Overall ANOVA shows significance \( F(3, 82) = 5.437, p \leq 0.01 \). Post-hoc analyses revealed that cold shock delivered immediately after each training block did not block long-term memory following distributed training \( t(45) = 2.52, p \leq 0.05 \). As expected, cold shock had no effect on the lack of memory seen at 24-hours after massed training \( t(37) = 0.318, p \geq 0.50 \). The results from this experiment show that while heat shock is an effective tool to inhibit protein synthesis and block memory in *C. elegans*, cold shock is not.

Using heat shock, I replicated the results seen in Beck and Rankin (1995) by showing that heat shock administration blocked memory observed 24 hours following distributed training in the between-groups training protocol. Heat shock can now be used
with this more rapid protocol to further explore the characteristics and mechanisms of long-term memory in C. elegans.

*Experiment 1B. Shortening the Inter-Block Interval Decreased Long-term Memory*

Beck and Rankin (1995) showed that heat shock effectively blocks long-term memory formation only when it is administered during the first 30 minutes of the one-hour rest periods. With this in mind we tested whether we could further shorten the training procedure with the new group-training protocol by running a distributed training group with only 40 minute rest intervals between blocks of training. When the inter-block interval was only 40 minutes there was no significant long-term memory for distributed training as the average response magnitude for the trained group was not significantly different from the average control response (mean ± standard error for 18 trained worms was 168.82 ± 19.45, for 21 tap control worms was 196.08 ± 16.53; t(37) = 1.08, p=.29 NS). Therefore, in all future experiments using this procedure, the inter-block interval must be at least 60 minutes.

*Experiment 1C. The New Group Protocol Shows an Accumulation of Memory With Increased Training*

Using nonsense word lists with human subjects, Ebbinghaus (1885) demonstrated that information is remembered longer if it is studied in blocks across time rather than all at once. The degree to which information was retained corresponded with the number of learning trials; greater retention occurred as the number of learning trials increased. As we have shown, in C. elegans only distributed training results in long-term memory 24
hours after training. If the principles outlined by Ebbinghaus apply to long-term memory for habituation in the worm then as more training blocks are included in the training procedure a greater degree of long-term retention should be produced. To test this four groups of worms received either 1 (n=23), 2 (n=22), 3 (n=20) or 4 blocks (n=27) of 20 single taps at a 60s ISI all separated with one hour rest periods. Each experimental training group was coupled to a single-tap matched control group (n= 22, 22, 17 and 27 respectively).

![Graph showing memory accumulation with increased training blocks](image)

**Figure 6.** Memory appears to accumulate as an increased number of training blocks are added. Mean response magnitudes of test phase for trained groups that received 1, 2, 3 or 4 blocks of training (white bars) compared to their respective single-tap matched control groups (black bars) shows that as amount of training increases the mean response magnitude decreases for the trained group thus suggesting greater long-term retention. Mean response magnitudes are expressed as percent control group response. * = p < 0.05.

Figure 6 shows the standardized mean reversal response magnitudes for the 10 test single taps for experimental and control groups and illustrates the graded increase in retention that was observed as the number of training blocks increased. An overall
ANOVA was significant (F(7, 172) = 2.941, p≤0.01). Planned comparisons with t-tests with the alpha level corrected for multiple tests, found that neither one, two nor three blocks of habituation training produced significant long-term memory (t(43) = 1.326, p>0.10, t(42) = 1.592, p>0.10 and t(35) = 1.618, p>0.10, respectively); however, four blocks of habituation training produced the greatest difference between the trained group and the matched single-tap control (t(52) = 3.076, p<0.01). These results demonstrate that memory accumulates as the number of training blocks increases. As well, this experiment supports the conclusion that the four blocks used in the new group-training protocol is an optimal balance between the time taken in training and the amount of long-term memory produced to make the new protocol an effective tool in exploring cellular mechanisms of long-term memory.

2.4 DISCUSSION

From these experiments it can be seen that the new group-training protocol for studying long-term memory for habituation in C. elegans is as effective as the original protocol reported in Beck and Rankin (1995; 1997). Both paradigms produced results that showed that distributed training resulted in long-term memory whereas massed training did not, that long ISIs produce long-term memory whereas short ISIs did not, and that heat shock during training blocked long-term memory for habituation. Because groups of worms can be tested within days (as opposed to weeks and months with the original single worm protocol), the new group-training protocol provides a faster, alternative method with which to examine long-term memory effectively.
Many researchers using other organisms have reported that learning is retained for longer periods of time as subjects are exposed to more and more blocks of training during the learning phase (i.e., Tully and Quinn, 1985). When the degree of long-term memory was examined across groups that received 1, 2, 3 or 4 blocks of training it is evident that the amount of memory gradually increased as another block of training was added. Massed training did not produce long-term memory, thus it appears that the rest periods between training blocks are important for memory formation. Like Beck and Rankin (1995) I report that heat shock administered during the rest periods between training blocks disrupts long-term memory formation. Heat shock produces a sudden yet sustained state of stress in an organism that causes all non-essential protein synthesis to cease including *de novo* protein synthesis thought to underlie long-term memory (Schlesinger et al., 1982; Lindquist, 1986). The disruption of long-term memory formation by heat shock suggests that some sort of consolidation of memory occurs during the one hour between training blocks and that this consolidation process is dependent on protein synthesis.

Interestingly, cold shock did not block memory in *C. elegans*. Cold shock has been shown to inhibit certain forms of memory in both *Drosophila* (Tully et al., 1994) and *Lymnaea* (Sangha et al., 2003b). Normally, when an organism experiences very low temperatures, cells begin to produce cold shock proteins. This process has been shown to be conserved from bacteria to man (Wolffe et al., 1992). However, it is possible that cold shock does not operate in the same fashion in *C. elegans*. *C. elegans* can survive freezing; *C. elegans* larvae can withstand freezing to a temperature of -80°C thus perhaps the cold-protective mechanism that allows for survival during freezing is maintained through
adulthood thus making worms less vulnerable to the effects of cold shock on memory formation. Based on these experiments I concluded that cold shock is not a useful tool for the study of long-term memory in *C. elegans*.

The results from the accumulation experiment suggest that at least three blocks and/or two rest periods are necessary for this protein synthesis to occur. The length of the rest periods is also important; when the rest period was shortened to 40 minutes, long-term memory for habituation was not observed. Although Beck and Rankin (1995) suggested that the first 30 minutes post-training was critical for protein synthesis the results from this experiment suggest some additional function for the second half of the 60-minute rest period. It would also be interesting to investigate the maximum duration of the rest period that still allows long-term memory for habituation to develop.

Taken together these experiments demonstrated that the new protocol that I developed produced reliable, protein synthesis dependent long-term memory in *C. elegans*. With this protocol in hand, and the identified circuit for tap habituation (Wicks and Rankin, 1995; see figure 2 in Chapter One), I then used this protocol to investigate mutations in genes involved in neurotransmission in the tap withdrawal circuit with the objective of discovering mechanisms of long-term memory for habituation in *C. elegans*. 
CHAPTER 3

Objective 2: The role of glutamate in long-term memory in C. elegans.

3.1 INTRODUCTION

Glutamate receptor trafficking has been hypothesized to be a mechanism of plasticity in the mammalian brain; conditions that increase neuronal communication can sometimes produce an increase in AMPA-type glutamate receptors while conditions that depress neuronal communication often produce a decrease in AMPA receptors (for reviews see Lüscher and Frerking, 2001; Malinow and Malenka, 2002). Many researchers hypothesize that these changes in plasticity underlie memory. Recently, using rats Rumpel et al. (2005) showed that learning a cued-fear conditioning task (tone-shock association) resulted in the incorporation of GFP-tagged GluR1 (the mammalian homologue of C. elegans GLR-1) in the amygdala (a brain area important for fear conditioning). Further, they found that if AMPA-type glutamate receptor (containing the GluR1 subunit) incorporation was blocked, this not only blocked LTP (see Chapter One) but this also blocked memory formation of fear conditioning. This suggests that changes in AMPA receptor expression may underlie both LTP and the behavioral expression of memory. In this series of experiments I report that glutamate and more specifically, that AMPA-type glutamate receptors are critical for long-term memory for habituation in Caenorhabditis elegans. As well, I demonstrate that AMPA-type glutamate receptor expression is altered as a function of training. This study also tested the efficacy of the new protocol in uncovering mutations that affect long-term memory in C. elegans by testing for long-term memory using both the old and new protocols (see Chapter Two)
with a mutant strain, *eat-4*, which shows altered short-term habituation (Rankin and Wicks, 2000).

*C. elegans* shows protein synthesis-dependent retention of habituation of the tap withdrawal response 24 hours after distributed training (Chapter Two; Beck & Rankin 1995; Rose *et al.*, 2002). The primary elements of the neural circuit for tap are 5 sensory neurons and 4 pairs of interneurons (as well as a large number of motor neurons (Chalfie, *et al.* 1985; Wicks & Rankin, 1995). There is evidence from studies of identified mutations expressed in pre- and post-synaptic neurons in the tap-withdrawal circuit that the sensory to interneuron synapses in the tap withdrawal circuit in *C. elegans* are glutamatergic (Hart *et al.*, 1995; Maricq *et al.*, 1995) therefore it is of interest to determine whether a glutamate deficiency will result in a deficit in long-term retention of the habituated response. This would provide evidence for a putative neurotransmitter involved in long-term memory.

The *eat-4(ky5)* gene mutation, originally studied by Avery (1993), has been characterized as a putative glutamate vesicular transporter and plays an important role in the transport of glutamate into presynaptic vesicles of glutamatergic neurons (Lee, Sawin, Chalfie, Horvitz and Avery, 1999) thus it is hypothesized to positively regulate levels of glutamate in the presynaptic neurons (Lee *et al.*, 1999). As well, *eat-4(ky5)* has been reported to be expressed in the mechanosensory neurons of the tap-withdrawal circuit (ALM, AVM and PLM; Lee *et al.*, 1999; see figure 7).

Because *eat-4(ky5)* is thought to code for a vesicular transporter it has been hypothesized that *eat-4(ky5)* mutant worms have a decreased availability of glutamate in
the presynaptic terminal (Rankin and Wicks, 2000). Previous behavioral studies involving eat-4(ky5) conducted by Rankin and Wicks (2000) have shown that the eat-4(ky5) mutant remains capable of responding normally to a single tap stimulus yet displays enhanced short-term habituation of the tap-withdrawal response.

Figure 7. Illustration showing gene expression in the tap-withdrawal neural circuit. Presynaptic mechanosensory neurons express the eat-4 gene (rectangles) while postsynaptic interneurons express the glr-1 gene (circles). Hatched lines denote electrical gap junctions between neurons while black arrows indicate chemical connections. Triangles represent motor neuron pools whose stimulation results in either worm reversal response (REV) or forward locomotion (FWD).

This was presumed to be due to the eat-4(ky5) mutant strain's inability to produce sustained glutamate release from the presynaptic terminal thus the eat-4 reversal response magnitude decreases more rapidly during habituation training compared to wild-type.

Worms carrying mutations in the eat-4 gene show slower spontaneous recovery (when response levels increase back to baseline following short-term habituation) and a deficit in dishabituation (where application of a novel or noxious stimulus return response levels to a formerly habituated stimulus back to baseline levels). To test how decreased
glutamate availability affected the formation of long-term memory, worms of the \textit{eat-4(ky5)} mutant strain underwent the group distributed habituation training protocol developed in the experiments described in Chapter Two. With this protocol, only the amount of training will differ between groups, thus controlling for any extraneous effects due to the mutation itself and/or specific environmental conditions on behavior.

To support the long-term memory findings for the \textit{eat-4} mutant strain, I also examined an \textit{eat-4} transgenic rescue strain (DA1242) to determine whether it was the \textit{eat-4(ky5)} mutation specifically that produced any deficit in long-term memory and not some secondary mutation associated with the \textit{eat-4(ky5)} mutant strain. The DA1242 strain was originally described by Lee \textit{et al} (1999) and was found to restore not only the feeding deficits originally reported for \textit{eat-4(ky5)} worms, but also the behavior changes reported by Rankin and Wicks (2000). The transgenic \textit{eat-4(ky5)} rescue strain (DA1242) was given long-term memory training using the new group-training protocol.

I also examined several glutamate receptors that might play a role in mediating long-term memory. Much research in mammalian nervous systems has focused on both NMDA-type and non-NMDA-type glutamate receptors as being key players in long-term synaptic plasticity in mammals, however, the activity of the NMDA receptor is generally considered to be initiated by repetitive activation of non-NMDA receptors (i.e., AMPA receptors). As well, there is now evidence that specific AMPA receptors may be associated with the formation of the gene transcription protein CREB (Perkinton \textit{et al}., 1999) which is critical for long-term memory formation (Kogan \textit{et al}., 1997; Wood \textit{et al}., 2005).
In *C. elegans* genes have been isolated that are expressed on the interneurons of the tap withdrawal circuit that code for three classes of glutamate receptors, *glr-1* (homologous to AMPA type; Hart, Sims, and Kaplan, 1995; Maricq, Peckol, Driscoll, and Bargmann, 1995), *avr-15* and *avr-14* (glutamate-gated Cl-channels; Dent et al., 1997; Dent et al., 2000) and *nmr-1* (homologous to NMDA-type channels; Brockie, et al., 2001). Pilot experiments on a strain of worms carrying a mutation in an NMDA-type glutamate receptor subunit and on strains of worms with a mutation in glutamate-gated chloride channels, *avr-14* and *avr-15*, showed normal long-term memory (see Appendix). Therefore I asked whether worms carrying mutations in the *glr-1* subunit homologous to mammalian AMPA-type glutamate receptors would also show normal long-term memory. *glr-1*(n2461) was originally isolated by Hart *et al.* (1995) and *glr-1*(kyl 76) was originally isolated by Maricq *et al.* (1995): both groups concluded that the *glr-1* gene was ~40% homologous to mammalian AMPA-class receptors. The *glr-1* is expressed on the interneurons that mediate the tap-withdrawal response (AVA, AVB, AVD and PVC; see figure 7). If it is found that long-term memory does not occur in the absence of AMPA receptors, this would support that AMPA is an upstream effector of any long-term memory mechanism.

To support any findings in regard to the role of glutamate in long-term memory, a pharmacological experiment was also conducted. For this experiment, worms received training in the presence of a competitive non-NMDA-type glutamate receptor antagonist 6,7-Dinitroquinoxaline-2,3-dione (DNQX). Worms were transferred and the 24-hour delay period and testing phase occurred in the absence of drug. This is a less precise method for examining the role of glutamate on memory as the internal concentration of
drug is determined by the amount of drug that enters the worm from the environment. Nevertheless, the results from this study should confirm any deficits in memory seen with strains carrying mutations that affect glutamate transmission.

Once I determined the effects of glutamate availability and a non-NMDA-type glutamate receptor mutation on long-term memory, my next step was to use transgenic green fluorescent protein (GFP) strains to examine how expression of particular genes change as a result of long-term memory. GFP, originally isolated from jelly fish, is a genetic construct that can be attached to a gene such that the protein products of that gene will glow green under fluorescent light (Chalfie et al., 1994). I examined two GFP transgenic strains (GLR-1::GFP and pmec-7::SNB-1::GFP) with confocal microscopy. Confocal microscopy allows for better control of depth of field and greatly reduces background fluorescence from the image resulting in a more precise measure of GFP expression.

The GLR-1::GFP strain is expressed in the interneurons of the tap-withdrawal circuit. The processes of these neurons are found in the ventral nerve cord where they make their connections. This anatomical organization makes GLR-1::GFP easily measureable along this process. Any increases or decreases in GLR-1::GFP expression will be indicative of more or fewer glr-1 receptor subunits being expressed (respectively) and thus would suggest a change in receptor number. I subjected GLR-1::GFP transgenic worms to long-term memory training and then measured GFP expression 24 hours after training to determine whether any stabilized changes had occurred as a result of training. A change in GLR-1::GFP in only trained worms would suggest a post-synaptic memory mechanism.
A final experiment was designed to demonstrate whether long-term memory produces presynaptic modulation such as a change in number or density of presynaptic vesicles. To accomplish this I performed confocal imaging with the \( \text{p}^\text{mec-7::SNB-1::GFP} \) transgenic strain 24 hours after long-term memory training. The SNB-1 codes for the vesicle membrane protein synaptobrevin so changes in \( \text{p}^\text{mec-7::SNB-1::GFP} \) expression should be indicative of presynaptic changes in vesicles. However, since all neurons contain vesicles to allow for chemical signaling, the \( \text{p}^\text{mec-7::SNB-1::GFP} \) strain has a \text{mec-7} promoter gene region meaning that only the vesicles of the mechanosensory neurons show GFP expression. I restricted examination of \( \text{p}^\text{mec-7::SNB-1::GFP} \) to the terminals of the PLM tail neurons that show a small area of expression just posterior to the vulva of the worm (Nonet, 1999). Any change in the number or size of fluorescing clusters would suggest the hypothesis that memory relies on a presynaptic mechanism.

Taken together the experiments in this chapter will confirm glutamate as a critical neurotransmitter underlying memory for long-term habituation and will provide strong evidence for a potential mechanism and locale of long-term memory formation.

### 3.2 METHODS

**Animals**

Worms were maintained on Nematode Growth Medium (NGM) agar seeded with \textit{E. coli} (OP50; Brenner, 1974). N2 \textit{C. elegans} Bristol (N2) and \textit{glr-1} (n2461) were obtained from the \textit{Caenorhabditis} Genetics Center. The \textit{eat-4} mutant strain and the \textit{eat-4} transgenic rescue strain DA1242 were originally obtained from Dr. Leon Avery at the University of Texas Medical School. \textit{eat-4(ky5)} is a loss-of-function, putative null allele
of a glutamate vesicular transporter (Avery, 1993). Lee, Sawin, Chalfie, Horvitz and Avery (1999) showed that the eat-4 gene rescuing activity was localized to a 6.9 kb region of cosmid ZK512. The strain (DA1242) was made using cosmid rescue with cosmid ZK512 that restored the wild-type feeding phenotype. Rankin and Wicks (2000) showed that DA1242 rescued the short-term habituation deficits seen in eat-4 worms. glr-1(ky176) worms were obtained from V. Maricq; GLR-1::GFP from J. Kaplan and mec-7::SNB-1::GFP from M. Nonet.

Behavioral Procedures

Both the original within-groups training procedure and the new between-groups training procedure were used in these experiments. If the original training protocol was employed then the training phase occurred with the microscope stage light on to allow for video-recording of responses. In the new between-groups protocol the training was not recorded therefore the training phase occurred without the microscope stage illuminated. Testing for both protocols occurred with the stage light on and was video-recorded. The original distributed training protocol for long-term memory for habituation training (described in detail in Beck and Rankin, 1997) consisted of an experimental group in which individual 4-day-old worms received 3 blocks of training. Each block consisted of 20 trains of taps given at 60s ISI. Training blocks were separated by one-hour rest periods on day 1, and a single block of 20 trains of taps was given 24 hours later on day 2. To test for long-term memory for habituation the mean reversal magnitude to the first block of trains of taps on day 1 was compared to the mean reversal magnitude for the block of trains of taps on day 2. A significantly smaller mean response on Day 2 was considered
to reflect long-term memory for habituation training. With this procedure training and testing of 20 worms took approximately 67 hours.

Habituation stimuli (taps/trains) were delivered using a Grass S88 stimulator driving a mechanical tapper exerting 1-2 N of force to the side of the petri plate. For studies of short-term habituation 20 single tap or train (train = 6 taps/sec) stimuli were delivered to single worms on blank NGM agar plates at a 60 s ISI. For studies of long-term memory for habituation groups of 10-15, 3-day-old worms were placed on E. coli seeded Petri plates 12-18 hours prior to training. On training day, experimental worms were given distributed habituation training (4 blocks of 20 taps or trains at a 60 s ISI separated by one-hour rest periods): control worms were given a single tap or train. One hour after training all worms were transferred to individual E. coli streaked plates. During testing (24-28 hours after training) each worm received 10 taps or trains (whichever was the same as that worm's training) at 60s ISI. The responses to the 10 test taps were scored and used in the data analysis. In studies where heat shock was used plates were sealed with parafilm and submerged in a water bath at 32 degrees Celsius for 40 minutes during the rest period between training blocks. Sham heat shock involved submerging plates in a water bath at 21 degrees C in the same procedure.

For studies on the role of DNQX on long-term memory the drug was dissolved in 35 mM NaOH to prepare 10 mM DNQX. Pilot experiments showed no differences in the reversal behaviors of worms treated with different dilutions of DNQX therefore a high drug concentration was used to ensure drug penetration. Two hours before training 500 ul of either 10 mM DNQX or 35 mM NaOH (vehicle) was placed onto the surface of agar plates bearing E. coli thus allowing enough time for drug-containing liquid medium to
dry on the surface of the agar. For the drug groups the 500 µl of 10mM DNQX applied to 10mL of agar made the effective external concentration in the worm’s environment 476µM. A previous study in the lab found that after one hour of exposure to 10 mM DNQX, wild-type worms responded to tap with reversals of a similar magnitude as the glr-1 mutant strain suggesting non-NMDA-type glutamate receptors had been effectively blocked. Therefore, one hour prior to training 4 groups of 10-15 4-day-old wild-type worms were transferred to drug-treated plates as one hour was presumed to be a sufficient amount of exposure time for the drug to have taken effect. This rationale is supported by pilot studies using toxic concentrations of DNQX that found Groups of worms were given either taps in the distributed training protocol at a 60 s ISI or the single tap control, and then 1 hour later transferred to individual drug-free plates for testing with 10 taps (at a 60 s ISI) the next day. Drug exposure was <6 hours and all testing was conducted ≥20 hours after removal of drug.

All behavioral data was scored using stop-frame video analysis to trace the distance traveled backwards to each tap or train onto acetate sheets. Tracings were scanned into the computer and measured using NIH image software. Occasionally worms swam rapidly forward in response to tap: these accelerations forward are qualitatively different from reversals and were scored as missing data points (fewer than 10% of the data).

Imaging Procedures

For imaging GFP transgenic worm strains were mounted onto welled slides using 12ul of 2,3-butanedione monoxime for paralysis mixed with medium Sephadex beads to prevent worms from being crushed. A Nikon Optiphot-2 microscope with an MRC 600
Confocal system (Bio-Rad) equipped with a Krypton/Argon laser was used for imaging. Green fluorescent protein (GFP) was excited using a 488nm wavelength laser setting with emitted light collected passing through a ~510-550nm bandpass filter. Images were captured in a 768X512 pixel field of view with optical sections collected at 0.5 um intervals using a 60x oil lens.

Twenty four hours prior to imaging GLR-1::GFP and \( \mu \text{mec-7}::\mu \text{SNB-1}::\mu \text{GFP} \) worms were given taps in the distributed training protocol or the single tap control. One hour after training worms were transferred to individual plates for imaging 24 hours later. The GLR-1::GFP strain expressed GFP along the ventral cord and images were collected along the posterior portion from the tail to the vulva. Images of GLR-1::GFP expression were composed of 10-15 optical sections. A researcher blind to the treatment groups measured images. Collected projection images were viewed and measured using NIH Image 1.61. GFP expression was measured as the length of GFP clusters as ventral cord width was relatively uniform across animals.

GFP expressed in the \( \mu \text{mec-7}::\mu \text{SNB-1}::\mu \text{GFP} \) worms was captured in a single projection image composed of approximately 12-18 optical sections. A researcher blind to the treatment groups measured images. In NIH Image a threshold adjustment produced high-contrast images in black and white to allow for viewing of faint GFP. Area measurements for each region of GFP expression were calculated by outlining the GFP expressing region and using the area measure function in NIH Image. Final figures were generated using Adobe Photoshop 7.0 to increase resolution of images.

Data Analyses
For experiments that compared trained worms to untrained controls, individual t-tests were used for comparison. Where several groups of control vs. trained worms were examined, an ANOVA was performed followed by planned comparison t-tests with a Bonferroni adjustment of the alpha level for multiple t-tests (significance was calculated by 0.05/number of t-tests). In graphs, mean reversal magnitudes of trained worms were standardized and expressed as 'percent control response'. In the single-worm protocol two-tailed paired t-tests were used to compare the average response from the first block of training to the average response from the test block on day 2.

3.3 RESULTS

Experiment 2A. Presynaptic Glutamate Release is Required to Produce Long-term Memory

I examined the role of presynaptic glutamate release in the formation of long-term memory for habituation training by studying eat-4 worms. The mammalian homologue of EAT-4 is a vesicular glutamate transporter and in C. elegans EAT-4 is expressed in the sensory neurons of the tap withdrawal circuit (Lee et al. 1999). The eat-4 mutation does not disrupt the worm’s ability to reverse spontaneously or in response to a single tap (Rankin and Wicks, 2000). However, in a study of short-term habituation with both short and long ISIs, worms with a loss-of-function, putative null mutation in eat-4 showed more rapid habituation to tap and slower recovery from habituation than did wild-type worms (Rankin and Wicks, 2000). If the deficit in sustained glutamate activity that was seen in short-term habituation negatively affects memory formation it would strongly suggest that glutamate transmission is necessary for long term memory for habituation
training in the tap withdrawal circuit. In the first experiment, *eat-4*(ky5) worms were tested using the original single-worm protocol with three blocks of trains of taps at a 60 s

Figure 8. With trains of taps *eat-4* mutant worms show significant short-term accumulation of habituation across blocks of training and a trend towards 24-hour retention. A) The mean response magnitude, standardized by worm length, for each train stimulus is graphed across all 20 stimuli in each of the three training blocks and across the 20 stimuli of the test block that occurred 24 hours later. B) Average reversal magnitude for each training block on Day 1 and the test phase on Day 2. Difference between bars indicated by line is p=0.12.
In this protocol it is possible to examine the acquisition of memory by looking at performance during training. Figure 8A shows the average response magnitudes for reversals in the three blocks of training on day 1 and the test block on day 2. When stimulated with trains of taps the eat-4 worms showed normal short-term habituation over each block of stimuli. In fact, with trains of taps eat-4 worms do not show the rapid and complete habituation reported by Rankin and Wicks (2000) for single taps. In addition, as can be seen Figure 8B, there was a steady accumulation of short-term habituation over the course of the three training blocks on day 1 (similar to that reported in wild-type worms by Beck and Rankin, 1997). When the mean response magnitude for the first block of training was compared to the mean of the test block on day 2 there was no statistically significant long-term memory for habituation, however there was a trend for smaller responses on the test day ($t(18) = 1.64, p \leq 0.12$). As well, day 2 habituation appears to be more rapid compared to the habituation rates on day 1 with the largest drop in reversal magnitude occurring between stimuli 1 and 2; this more rapid rehabituation may reflect memory for the earlier training (Figure 8A).

In a second experiment eat-4 worms were tested using the new group-training method where the distributed group received four blocks of single taps. ANOVA on the mean reversal magnitudes to the 10 test single taps 24 hours after training was significant ($F(5, 124) = 16.142, p < 0.0001$). Planned comparisons showed that the eat-4 worms showed no evidence of long-term memory for the habituation to tap training ($t(42) = 0.378, p \geq 0.500$; Figure 9).

In order to verify that it was the eat-4 mutation that produced the deficit in long-term memory following training with single taps and not some secondary mutation
associated with the eat-4 mutant strain, a transgenic eat-4 rescue strain (DA1242 in which a sequence including the eat-4 gene has been added back into eat-4 worms) was

![Graph showing response magnitudes for each trained group (white bars) expressed as percent control response (black bars).](image)

**Figure 9.** With the new group-training protocol 24-hour retention occurs with eat-4 worms only if a strong stimulus (trains) is applied. The mean response magnitudes for each trained group (white bars) are expressed as percent control response (black bars). When the weaker tap stimulus is used for training, no 24-retention results when compared to the matched control group. The eat-4 rescue strain (DA1242), where the eat-4 mutant is rescued by a wild-type transgene, shows long-term memory for habituation with single taps rescuing the deficit. If a stronger stimulus (trains) is used with the new group-training protocol long-term memory for habituation is also produced. * = \( p < 0.05 \).

given distributed training using the new group-training protocol with single taps. The DA1242 strain was originally described by Lee *et al* (1999) and, in behavioral assays, was found to restore not only the feeding deficits originally reported for eat-4 worms, but also the change in habituation reported by Rankin and Wicks (2000). Planned comparisons with the DA1242 showed that the trained group showed significantly smaller responses than the did the single-tap controls \((t(48) = 2.745, p \leq 0.005; \text{Figure 9})\).

This indicated that the eat-4 transgene successfully rescued the long-term memory for
habitation training with single taps and that the deficit seen in *eat-4* worms was the result of a mutation in *eat-4* and not in some other unknown gene.

One major difference between the original single-worm protocol and the group-training protocol is that the original protocol used trains of taps as the stimulus, while the new protocol used a single tap as the stimulus. Rankin and Wicks (2000) reported very rapid habitation in *eat-4* worms with both short and long ISIs using the single tap stimulus. In contrast, in the single-worm protocol when trains of taps were delivered at a 60 s ISI the rate of habituation over 20 stimuli for *eat-4* worms was only marginally faster than what we see in wild-type worms (i.e., Beck and Rankin, 1997). Thus, it appeared that *eat-4* worms showed less of an alteration in short-term habituation to trains of taps than to single tap stimuli. When the results from the original protocol with three blocks of 20 trains of taps (t(18) = 1.64, p = 0.12) are compared to the results of the new protocol with four blocks of 20 single taps (t(42) = 0.38, p=0.71) it appears as though the trains of taps might be producing some memory while it is clear that the single taps are not. To test whether trains of taps could produce long-term memory for habituation the new group-training protocol was given using four blocks of trains of taps rather than single taps. Planned comparisons with the new group-training procedure with the stronger train of taps did result in significant long-term memory for habituation training in the group of *eat-4* mutants that received distributed training (t(33) = 2.736, p<0.01) when compared to the single train control group (Figure 9). Thus, if sufficient stimulation is given then *eat-4* worms will show long-term memory for habituation training.

*Experiment 2B. Behavioral Analyses of glr-1 in Long-Term Memory*
Both of the *glr-1* alleles (ky176 and n2461) were originally hypothesized to lead to null mutations (Hart *et al.*, 1995; Maricq *et al.*, 1995) however, when I looked at the responses of worms to mechanosensory stimuli there were differences between the strains in response magnitude to different stimuli. *kyl76* showed almost no reversal to tap, while *n2461* showed reversal responses to tap similar to wild-type worms (response to tap in pixels for *N*=20 *glr-1*(n2461) worms was Mean = 220.03 ±49.18, while for *N*=10 *glr-1*(ky176) worms 5.95 ±4.04). Both strains reversed in response to a stronger stimulus, a train of taps (response to train for *N*=19 *glr-1*(n2461) worms: Mean = 744.64 ±50.59, and for *N*=16 *glr-1*(ky176) worms: 542.88 ±65.00). These observations suggest that the truncated protein produced by *kyl76* is not totally lacking in function. Because *glr-1*(ky176) did not respond to single taps but did respond to trains of taps, *glr-1*(n2461) was tested with both trains and taps, while *glr-1*(ky176) was only tested with trains.

**Figure 10. Mutations in *glr-1* do not impair short-term habituation at a 60s ISI.**
Mean response magnitude in pixels for a) wild-type, and b) *n2461* worms given 20 taps at a 60 s ISI (*ky176* does not respond to taps). Data are presented averaged into 10 bins of 2 responses each. Both groups show a similar gradual decrease in response magnitude with repeated stimulation. Mean response magnitude in pixels for c) wild-type, d) *n2461* and e) *kyl76* worms given 20 trains of taps (1 train = 6 taps within 600ms) at a 60 s ISI. Data are presented averaged into 10 bins of 2 responses each, as above. All groups show a similar gradual decrease in response magnitude with repeated stimulation. Paired comparisons between bin 1 (average response to the first two stimuli) and bin 10 (average response across the last two stimuli) were significant in all cases with adjustment for multiple comparisons (N2 taps *t*(19)=4.73, *p*<0.01; *n2461* taps *t*(19)=3.21, *p*<0.05; N2 trains *t*(17)=3.82, *p*<0.05; *n2461* trains *t*(18)=5.11, *p*<0.01; *ky176* trains *t*(15)=4.33, *p*<0.01).
Despite differences in initial response magnitude, both glr-1 strains showed significant short-term habituation to 20 stimuli at a 60 s interstimulus interval (ISI) (Figure 10) suggesting that the gene does not play a direct role in short-term habituation. To examine the role of glr-1 in long-term memory for habituation I used a between-groups distributed training protocol (training: 4 blocks of 20 stimuli at a 60 s ISI each separated by 1 hour rest periods; testing 10 stimuli at a 60 s ISI 24 hours after training) to test wild-type N2, n2461 and ky176 worms. On day 2, when I compared the test responses of trained wild-type worms to trained glr-1 mutant worms, I found N2 worms showed long-term memory for both trains and taps and neither glr-1 allele showed long-term memory for either taps or trains (Figure 11a). An ANOVA comparing the trained and control was significant ($F(5, 108) = 3.17, p<0.01$). Planned comparisons using t-tests with a corrected alpha level due to multiple comparisons showed that wild-type worms showed long-term memory in the form of significantly lower responses than controls when trained with both taps ($t(34) = 2.607, p<0.01$) and trains ($t(38) = 2.588, p<0.01$); none of the glr-1 trained groups differed significantly from controls ($p>0.05$ in all cases). In addition the responses of glr-1(n2461) trained worms were significantly larger than the responses of trained wild-type worms with both taps ($p=0.009$) and trains ($p=0.03$); and the responses of glr-1(ky176) trained worms were significantly larger than the responses of trained wild-type worms with trains ($p=0.04$). Together these data suggest the hypothesis that GLR-1 is required for the formation of long-term memory for habituation.
Experiment 2C. Pharmacological Blockade of non-NMDA type Glutamate Receptors

During Training Inhibits Long-term Memory Formation

To confirm the importance of glr-1 receptors in long-term memory wild-type (N2) worms were treated with the competitive non-NMDA glutamate receptor antagonist DNQX (6,7-Dinitroquinoxaline-2,3(1H,4H)-dione; Sigma). DNQX blocks neurotransmission at the Aplysia sensory neuron to motor neurons synapse (Dale & Kandel, 1993; Chitwood, Li & Glanzman 2001). DNQX does not block short-term homosynaptic depression (the cellular analogue of habituation; Armitage and Siegelbaum, 1998) but does block lasting memory for habituation in Aplysia (Ezzeddine & Glanzman, 2002). In C. elegans neither DNQX nor the vehicle control NaOH blocked significant short-term habituation to 20 taps at a 60 s ISI (t-test for mean of responses 1 & 2 vs. mean of responses 19 & 20 for NaOH t(11)=3.2, p=0.008; for DNQX t(11)=3.3, p=0.007).

N2 worms were given distributed training on agar plates treated with 35 mM NaOH (vehicle; N= 22; Figure 11b) or 10 mM DNQX (N= 27) dissolved in NaOH. Matched control groups (n= 21 for NaOH; N= 24 for DNQX) underwent the same drug treatments but received only 1 tap during the training phase. An ANOVA comparing trained DNQX and trained NaOH worms against untrained control worms was significant (F(2, 69) = 4.1, p≤0.02). Planned comparison t-tests showed significant differences between NaOH trained and control worms (t(42) = 2.077, p≤0.05) with no significant difference between DNQX trained worms and control worms (t( 51) = 1.201, p≥0.10).
Figure 11. The role of glr-1 glutamate receptors in memory. A) Mutations in glr-1 (results from 2 alleles, n2461 and ky176) eliminate long-term memory for habituation. Mean percent control response magnitude for long-term memory for habituation training of the tap withdrawal response in wild-type (WT) and glr-1(n2461) and glr-1(ky176). Average reversal magnitude across the test trial for trained groups (black bars) expressed as percent control response (top black line across graph; dotted lines represent ± average SEM for control groups). White numbers in bars represent the number of worms in each trained group. Both wild-type (WT) groups showed significant long-term memory; none of the glr-1 groups showed significant long-term memory to either taps or trains. B) Exposure to the non-NMDA glutamate receptor antagonist DNQX during training blocks long-term memory for habituation of the tap withdrawal response in wild-type worms, exposure to the vehicle control NaOH during training does not affect formation of long-term memory.

Thus, exposure to vehicle during training had no effect while exposure to vehicle plus DNQX during training blocked the development of long-term memory for habituation.

Experiment 2D. Training Alters GLR-1::GFP Expression

Taken together the experiments with the glr-1 worms and the wild-type worms exposed to DNQX show that the glr-1 receptor is critical for long-term memory of habituation in the tap withdrawal circuit. This suggested the hypothesis that habituation training might alter glr-1 expression. To investigate this possibility I tested worms carrying chimeric receptors made up of GLR-1 tagged with green fluorescent protein (GLR-1::GFP) to visualize changes in glr-1 expression (Rongo & Kaplan, 1999). In
GLR-1::GFP worms fluorescence is localized to clusters along the ventral cord neurites and in the nerve ring (corresponding to anatomical synaptic markers reported in EM studies; White, Southgate, Thomson, & Brenner, 1986).

Distributed long-term memory training produced behavioral long-term memory in the GLR-1::GFP transgenic worms \((t(44)=2.5, p=.02)\). Confocal imaging was used to visualize the GLR-1::GFP. I compared the amount of GLR-1::GFP expression in the ventral cord in trained worms and control worms. There were significantly smaller GFP clusters in trained worms (measured in pixels as total amount of GFP per worm; Mean = 280.95 ± 32.37; N=21) than in the single stimulus control worms (Mean = 463.70 ± 43.74; N=16; \(t(35)=3.44, p=0.002\)) although the number of clusters between groups did not differ (trained mean = 62.2 ± 7.14, control mean = 69.3 ± 10.3; \(t(35)=0.59, p\geq 0.50\)). Worms that received distributed training 24 hours earlier showed no difference in the number of GLR-1::GFP clusters in the ventral cord, however, the overall size of GFP clusters in the trained worms was significantly smaller than in the control worms (Figure 12a). These data led to hypothesize that long-term habituation training did not produce a change in the number of synapses but did reduce the number of \(glr-1\) receptors expressed per synapse on the interneurons.

To look for the presynaptic changes following training I measured GFP expression of the gene for \(C.\ elegans\) synaptobrevin (\(snb-1\)), a protein associated with synaptic vesicles, in the tap sensory neurons. SNB-1::GFP was expressed under the control of the \(mec-7\) promoter that restricts GFP expression to the six mechanosensory neurons of the tap circuit (Nonet, 1999). Distributed long-term memory training produced behavioral long-term memory in the \(\text{p}mec-7::\text{SNB-1}::\text{GFP}\) transgenic worms \((t(44)=\)
In these worms I focused on an accumulation of fluorescence in areas that corresponded with a specific set of tail-touch cell synaptic connections seen in EM studies (White et al., 1986). The fluorescence I measured appeared as one or two patches in the ventral cord just posterior to the vulva (Nonet, 1999). I measured the total area of the patches in the ventral cords of worms that received distributed training and control worms and found that there was no difference in the

Figure 12. Confocal images of GFP expression in trained and control worms. A) Confocal images of post-synaptic expression of glr-1 receptors on processes of the interneurons in the posterior ventral nerve cord visualized with GLR-1::GFP showing a single-tap control worm (control) and a worm that received distributed habituation training 24 hours earlier (trained; same procedure as Figure 10). Scale bar = 20 um. B) Confocal images of the vesicles in tap sensory neuron terminals visualized with a synaptobrevin GFP marker showing a single tap control worm (control) and a worm that received distributed habituation training 24 hours earlier (trained). Scale bar = 20 μm.
amount of SNB-1::GFP expression in the two groups (Mean = 41.00 ± 3.47; N=12) and
the single stimulus control worms (Mean = 42.5 ± 5.4; N=12; t(22)=0.41, p=0.69; Figure
12b). Thus long-term memory for habituation did not affect the expression of
synaptobrevin in these sensory neuron terminals.

Since the behavioral expression of long-term memory for habituation can be
blocked by exposure to heat shock in the one hour rest intervals between training blocks
(Beck & Rankin, 1997; Rose et al., 2002) I tested whether the heat shock would alter the
effect of training on the expression of GLR-1::GFP. I found that 24 hours after training
the mean length of GLR-1::GFP clusters in worms that received heat shock (32 degrees C
for 40 minutes after each training block) during distributed habituation training on day 1
did not differ from worms that received only a single tap on day 1 (trained with heat
shock N=18, Mean = 496.95 ± 50.2 pixels; control with heat shock N=16, Mean = 468.75
±54.26 pixels; (t(31)=0.380, p=0.71). In contrast, worms that received distributed
training with sham heat shock (21 degrees C for 40 m after each training block) showed
decreased mean length of GLR-1::GFP clusters 24 hours after training when compared to
their single tap controls (trained with sham shock N=18, Mean = 374.8 ± 26.38 pixels,
control with sham shock N= 18, Mean = 485.26 ± 33.47 pixels; t(34)=2.6, p=0.01). Thus
heat shock blocked the alteration in GLR-1::GFP expression usually seen following
distributed habituation training, without affecting any other aspect of GLR-1::GFP
expression (compare GLR-1::GFP levels in control heat shock and sham shock groups).
3.4 DISCUSSION

The synapse between the tap sensory neuron and the interneurons is hypothesized to be glutamatergic (Rankin and Wicks, 2000). When a mutation in eat-4, a vesicular glutamate transporter, was present the amount of glutamate available at the sensory neuron terminal was decreased and short-term habituation was very rapid compared to habituation in wild-type worms. In eat-4 worms, distributed training with a tap stimulus did not produce long-term memory. This suggested that sustained glutamatergic activity is necessary for long-term memory formation.

The observation that distributed training with stronger and increasingly more stimulation produced long-term memory progressively in eat-4 worms suggests that if enough strong stimuli are used sufficient glutamate is released from the sensory neuron to produce long-term memory for habituation training: 4 blocks of single taps produced no long-term memory whereas 3 blocks of trains of taps resulted in a trend towards long-term memory, but it took 4 blocks of trains of taps for eat-4 to start to show significant long-term memory.

The experiments in this chapter have examined long-term memory for habituation of wild-type worms and mutant eat-4 worms using either the original single-worm training procedure or the new group-training procedure. Both protocols produced comparable results thus supporting a more efficient way of examining the characteristics of long-term memory in C. elegans. The effect of increased stimulation leading to increased long-term memory for habituation training in eat-4 worms suggests that glutamate does participate in the formation of long-term memory in the worm.

In behavioral studies I showed that mutations in the excitatory glutamate receptor
gene \textit{glr-1} did not affect short-term memory for habituation in \textit{C. elegans}, but blocked long-term memory. A glutamate receptor antagonist also blocked long-term memory for habituation. Therefore, the receptor encoded by the \textit{glr-1} gene is hypothesized to play a critical role in long-term memory for habituation in the tap withdrawal response in \textit{C. elegans}. Interestingly, in the rat bilateral infusion of an AMPA-receptor antagonist immediately after training impaired long-term memory for habituation to a novel environment (Vianna, \textit{et al.}, 2000).

When I used confocal imaging to examine expression of GLR-1::GFP 24 hours following long-term memory training no decrease in the number of GFP clusters was observed, but there was a significant decrease in the overall amount of GFP expressed. This suggested that although there was no change in the number of synapses, there was a change in the density of receptors at the synapses. The observation that there was no change in the amount of synaptobrevin in the terminals of the sensory neurons suggests that training, and the decrease of post-synaptic glutamate receptors did not alter the number of vesicles in the pre-synaptic terminal. This was an unexpected result because in \textit{Aplysia} long-term memory for habituation has been correlated with a presynaptic decrease in the number of synapses, size of synapses and number of vesicles in active zones; no studies have looked post-synaptically (Bailey & Chen, 1983). One possible explanation for the differences is that in our experiments I used only a short training regimen that produces behavioral long-term memory; the \textit{Aplysia} received extensive training, much more than would be needed to produce behavioral changes. I have recently collected pilot data that suggests that if worms are over-trained there are decreases in the number of vesicles in the sensory neuron terminals (Rose and Rankin,
unpublished observations).

The observation that heat shock blocks the behavioral expression of long-term memory after distributed but not massed training has been used to support the hypothesis that the long-term memory observed at 24 hours post-training is protein synthesis dependent. Heat shock is thought to block on-going and new protein synthesis from occurring for a critical period of time after each training block (Beck and Rankin 1997; Rose et al., 2002). The finding that heat shock also blocks the change in GLR-1::GFP expression suggests that changes in GLR-1 expression due to memory is also protein synthesis dependent.

This is the first study to simultaneously examine both pre- and post-synaptic anatomical changes in the nervous system of living animals using the same stimulus protocol that produced behavioral expression of long-term memory. The data from these experiments support the hypothesis that long-term memory for habituation in C. elegans is mediated not by a change in the number of synapses, but by a down-regulation of non-NMDA-type glutamate receptors on the post-synaptic interneurons that weakens the strength of the sensory to interneuron synapses. This is consistent with data on AMPA receptor trafficking that is being reported in mammalian LTD preparations in which electrical stimulation or drug treatments that produce a weakening of synaptic strength also produce a down regulation of surface expressed AMPA receptors (reviewed in Lüscher and Frerking, 2001; Malinow and Malenka, 2002). Heynen et al., (2000) have suggested that memory is encoded by experience-dependent assignment of glutamate receptors to synapses. C. elegans offers an exciting in vivo system to study glutamate receptor trafficking as it relates to behavioral changes and experience.
CHAPTER 4

Objective 3: Long-Term Memory for Habituation is Not Permanent

4.1 INTRODUCTION

Reconsolidation of memory (the theory that retrieval of a previously consolidated memory returns part of the memory to a modifiable or labile state) has received much interest over the past five years. Using an associative fear conditioning task, Nader et al. (2000) reported that the protein synthesis inhibitor, anisomycin, administered post-reactivation induced amnesia for that consolidated memory. Many researchers have since investigated this phenomenon using associative learning tasks with both vertebrate (e.g., Wang et al., 2005; Taubenfeld et al., 2001; Eisenberg and Dudai, 2004) and invertebrate model systems (e.g., Sangha et al., 2003c; Merlo et al., 2005). However, to date no study has measured direct cellular changes resulting from the abolition of a consolidated memory. To this end, I examined the effects of reconsolidation blockade for a non-associative learning paradigm (i.e., habituation) in the model system Caenorhabditis elegans.

Similar to memory in other organisms, long-term memory for habituation (≥24 hours) requires a distributed training protocol, protein synthesis and intact glutamate transmission (Chapter Three, Rose et al., 2002; Rose et al., 2003). Expression of this memory is blocked when training occurs in the presence of the non-NMDA-type glutamate receptor inhibitor (DNQX) suggesting that non-NMDA-type glutamate receptor (e.g., AMPA) activation is necessary during training for long-term memory
formation suggesting that DNQX blockade of non-NMDA-type glutamate receptor activation during the training period interfered with the memory consolidation process. To determine if non-NMDA-type glutamate receptor activation also played a role in re-establishing a previously consolidated memory, therefore, I examined if long-term memory was affected if worms were exposed to DNQX during a reminder.

Long-term memory for habituation in *C. elegans* has also been correlated with a decrease in GLR-1::GFP expression in the command interneurons for the response (see Chapter Three; Rose *et al.*, 2003). Given that one of the cellular mechanisms mediating memory consolidation (post-synaptic decrease of *glr-1* containing receptors) can be quantified with this paradigm, I was in the unique situation of being able to test whether reconsolidation occurs and if it does, measure cellular changes associated with abolished long-term memory resulting from blockade of reconsolidation.

### 4.2 METHODS

**Animals**

Worms were maintained on nematode growth medium (NGM) agar seeded with *Escherichia coli* (OP50) (Brenner, 1974). N2 *C. elegans* Bristol (N2) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN), GLR-1::GFP from A. Hart (Harvard University, Boston, MA), and *p mec-7::SNB-1::GFP* from M. Nonet (Washington University, St. Louis, MO).

**Behavior**
Reconsolidation studies employed a modified version of a previously established long-term habituation training protocol using tap stimuli (Chapter Two; Rose et al., 2002; Rose et al., 2003; 4 blocks of 20 taps delivered at a 60s ISI with a one hour rest period following each training block) with reminder training (10 taps at a 60s ISI). Worms were transferred one hour after reminder onto individual \textit{E. coli} streaked plates. Testing occurred 46-50 hours after training consisting of 10 taps at a 60 sec ISI. Response measuring and quantification was performed as previously reported (Chapter Two, Rose et al. 2002, Rose et al., 2003).

\textit{Heat Shock}

Heat shock was performed using previously described methods (Chapter Two, Rose et al., 2002) following reminder training or at 24 hours after training in groups where no reminder was given.

\textit{Pharmacology}

For experiments involving 6,7-dinitroquinoxaline-2,3(1\textit{H},4\textit{H})-dione (DNQX) the drug was prepared as previously reported (Chapter Three, Rose et al. 2003). Two hours before reminder, 500 \textmu l of either 10 \text{mM} DNQX or 35 \text{mM} NaOH (vehicle) was placed onto the surface of agar plates bearing \textit{E. coli}. One hour before reminder (~24 hours after training), two groups of 10-15 4-d-old N2 worms were transferred to drug-treated plates and another two groups were transferred to vehicle-treated plates. All worms were given 10 taps at a 60 sec ISI and transferred one hour later to individual drug-free plates.
Testing occurred 48 hours after training. Drug exposure was <3 hr, and all testing was conducted ≥20 hr after removal of drug.

Imaging

Confocal imaging of GFP transgenic worm strains were performed in the same manner as published previously (Chapter Three, Rose et al., 2003), however, an inverted Zeiss Axiovert with DIC Optics microscope with a Bio Rad Radiance Plus confocal system (Bio-Rad, Hercules, CA) was used.

Data Analyses

Response magnitudes and measures of GFP expression were compared between groups using one-way ANOVA procedures. A Student Newman-Keuls test was employed for post-hoc analyses.

4.3 RESULTS

Experiment 4A: Blockade of Memory Reconsolidation Abolishes Long-Term Memory

The protocol for long-term memory for habituation is shown in figure 13A. To examine whether habituation memory was vulnerable to blockade of reconsolidation, one pair of control and trained groups received heat shock to arrest de novo protein synthesis immediately following reminder (10 taps at a 60s ISI). Heat shock has been reported to block ongoing protein synthesis in a number of studies (Duncan and Hershey, 1984; Panniers, 1994), and in C. elegans is preferable to drug treatment in that it can be tightly temporally regulated, and worms recover from its effects within 15 minutes. There were
significant differences in response magnitude between the four groups (Fig 13b, F(1,81) = 4.521, p≤0.006). Post-hoc tests showed that the trained group in the no shock condition responded significantly less than the matched control group while the control and trained worms that received heat shock responded with reversals of similar magnitude indicating

**Figure 13. Long-term memory for habituation is sensitive to reconsolidation blockade.** A) Reconsolidation protocol, trained worms received 4 blocks of 20 taps and 10 taps for reminder. Testing consisted of either measuring response to 10 taps or confocal imaging of GFP strains; B) Mean (±SEM) response during testing for control and trained worms that received reminder training followed by heat shock or no shock, C) Retention protocol; same as above but no reminder, D) Mean (±SEM) response during test for control and trained worms that received no reminder with heat shock or no shock given during the retention interval. *=p<0.05.

...that the long-term memory was no longer present. Groups tested 48 hours post-training with no reminder (figure 13c) still demonstrated retention shown by a significant ANOVA (F(3, 62) = 5.087, p≤0.003). Newman-Keuls post-hoc analyses revealed worms showed 48-hour retention (figure 13d) regardless of whether heat shock alone was delivered 24 hours after training or not. These data show that memory for the long-term
training for habituation lasts at least 48 hours, and is not disrupted by a heat shock delivered 24 hours after training. When a reminder treatment was given 24 hours after training it did not disrupt retention; in contrast when the reminder training was followed by heat shock the memory for the training at the 48-hour test was abolished. Thus, long-term habituation in *C. elegans* is sensitive to reconsolidation blockade.

**Experiment 4B: Memory Reactivation in the Presence of DNQX Also Interferes with Reconsolidation.**

In Chapter Three I showed that blockade of non-NMDA-type glutamate receptors with 10mM DNQX during training abolished long-term memory (Rose et al., 2003). In this study, I found that non-NMDA-type glutamate receptor blockade during memory reactivation (figure 14a) interferes with later retention (figure 14b; $F(3, 103) = 4.090$, $p \leq 0.01$). Newman-Keuls post-hoc tests showed that when vehicle (35 mM NaOH) was present during reminder there was still significant retention at 48 hours post-training demonstrated by trained worms responding with significantly smaller reversals compared to control worms, however, when DNQX was present during reminder there was no retention as both control and trained worms in the DNQX conditions responded to tap with reversals of a similar magnitude. When trained and control groups were exposed to either vehicle or DNQX with no reminder (figure 14c), significant retention was seen (figure 14d; $F(3, 67) = 6.103$, $p \leq 0.001$). Newman-Keuls post hoc analyses revealed a significant difference in reversal magnitude between control and trained groups that received vehicle exposure as well as between control and trained worms on DNQX (figure 14d). If GLR-1 receptor activation was required for memory retrieval, then DNQX would have had no effect on later retention as the memory would not have been
recalled successfully and presumably the memory would have remained intact. Because
the reactivation in the presence of DNQX led to the abolition of the memory it suggests
that glr-1 receptors are not necessary for the retrieval of the consolidated memory. In this

Figure 14. Reconsolidation requires non-NMDA-type glutamate receptor activation.
A) Drug reconsolidation protocol; both control and trained worms are exposed to either
DNQX or vehicle for the hour prior, during and the hour following reminder, B) Mean
(±SEM) response to tap during test for worms that received reminder in the presence of
either DNQX or vehicle, C) Drug retention protocol; worms are exposed to either DNQX
or vehicle for 130 minutes 24 hours after training, D) Mean (±SEM) response to tap
across the 10 test taps in worms that received no reminder in the presence of either
DNQX or vehicle. *=p<0.05.

case, in the presence of DNQX the electrical synapses would still have activated the
neural circuit for response to tap and activated the memory trace which was then sensitive
to reconsolidation blockade. These data suggest that, similar to the initial memory
process, reconsolidation of memory also requires the activation of non-NMDA-type
 glutamate receptors.

Experiment 4C. Reconsolidation Blockade Returns GLR-1 Expression to Baseline
In Chapter Three I reported a significant decrease in GLR-1::GFP expression 24 hours after long-term habituation training (Rose et al., 2003). In the current study I examined GLR-1::GFP expression 48 hours after training with a reminder treatment followed by either no shock or heat shock. There was a significant difference between groups (figure 15a; F(3, 38) = 3.248, p=0.032); worms that received a reminder treatment followed by no shock showed significantly less GLR-1::GFP expression than controls (figure 15a; p=0.004). However, if trained worms received heat shock following reminder training, this reset the level of GLR-1::GFP expression to control levels (p=0.972). There was no difference in number of clusters of GLR-1::GFP regardless of treatment (no shock following reminder, control Mean = 66.78 ± 4.52 versus trained Mean = 61.20 ± 5.33; heat shock following reminder, control Mean = 67.46 ± 5.14 versus trained Mean = 68.55 ± 5.85; F(3, 42) = 0.494, p=0.689) thus these changes in GLR-1::GFP expression are not due to a changing number of connections, but rather due to a modification in the amount of GLR-1::GFP expressed at each synapse. If worms were not given a reminder treatment there was significantly less GLR-1::GFP in trained groups than in control groups regardless of whether heat shock was administered 24 hours after training (figure 15b; F(3, 32) = 3.980, p=0.016). Thus, like the behavioral expression of long-term memory for habituation the decrease in GLR-1::GFP seen 24 hours after distributed habituation training is still present 48 hours after training. Heat shock delivered 24 hours after training did not disrupt the decrease in GLR-1::GFP seen at the 48 hour test. When a reminder treatment was given 24 hours after training it did not disrupt the decrease in GLR-1::GFP; in contrast when the reminder training was followed by heat shock the decrease in GLR-1::GFP measured at the 48-hour test was abolished and GLR-1::GFP
Figure 15. GLR-1::GFP expression is sensitive to reconsolidation blockade. A) Mean (±SEM) of total GFP expression imaged 24 hours after reminder training, B) Mean (±SEM) tally of GFP expressing clusters imaged in GLR-1::GFP worms that received no reminder training.

expression returned to baseline levels. Thus, the decrease in GLR-1::GFP following long-term habituation training in C. elegans is also sensitive to reconsolidation blockade.

Experiment 4D. Modulation of Memory by Blockade of Reconsolidation has No Effect on Synaptobrevin Expression.

In earlier studies of long-term memory for habituation (see Chapter Three) using a \textit{p}mece-7::SNB-1::GFP transgenic strain I showed that there was no presynaptic change correlated with memory (Rose \textit{et al.}, 2003). In the current study I found no difference in \textit{p}mece-7::SNB-1::GFP expression between trained worms and controls at 48 hours post-training (F(3, 39) = 2.186, p=0.105). I also found no difference in \textit{p}mece-7::SNB-1::GFP expression between control and trained groups that received reminder training (F(3, 42) = 1.506, p=0.227) indicating that neither consolidation nor reconsolidation involve long-lasting changes in presynaptic synaptobrevin.

4.4 DISCUSSION
The current studies extend my previous findings that distributed habituation training results in memory at 24 hours post-training (Rose et al., 2003), by demonstrating that this memory can be retained up to 48 hours following training. This 48-hour memory does not require reminder treatment. To examine whether memory for non-associative learning is subject to reconsolidation following reactivation, protein synthesis was blocked following reminder training. Similar to what has been reported for associative memory processes, inhibiting reconsolidation abolished expression of the previously established memory when measured 24 hours later. This is the first report of reconsolidation for a non-associative form of learning.

To distinguish whether reconsolidation processes rely on the same signaling pathways as long-term memory formation I examined the role of glutamate by exposing worms to a non-NMDA type glutamate receptor blocker (6,7-dinitroquinoxaline-2,3(1H,4H)-dione; DNQX) for the reminder period. In Chapter Three I demonstrated that when training occurred in the presence of DNQX, this inhibited long-term memory 24 hours later. Similarly, in this study I showed that when worms were exposed to DNQX during the reminder training period, this also attenuated the expression of memory 24 hours after memory reactivation. This suggests that, similar to the learning process, reconsolidation requires the activation of non-NMDA-type glutamate receptors. This demonstrates that the same glutamate-dependent plasticity required for long-term memory is also involved in establishing memory post-reactivation.

Similar to what I reported in Chapter Three (Rose et al. 2003), 48-hour memory for habituation also results in a significant decrease in GLR-1::GFP expression with no change in expression of the presynaptic vesicular marker \( p\text{mec-7}::\text{VAMP}::\text{GFP} \). This
decrease in GLR-1::GFP expression was shown to be sensitive to attenuation of protein synthesis following memory reactivation meaning that the original reorganization of GLR-1::GFP seen with long-term memory was essentially reset if reconsolidation was inhibited after memory reactivation. The findings of this series of experiments confirms the observation that GLR-1::GFP density is closely correlated with the expression of memory for habituation. This is the second report of changes in GLR-1::GFP expression produced by the same behavioral training protocol and on the same time scale as the noted behavioral changes (Rose et al., 2003). Together these results suggest that the expression of memory at 48 hours post-training involves the same or similar mechanisms as what occurs for previously reported 24-hour retention.

However, it remains to be determined whether GLR-1 density is in and of itself the mechanism of memory or whether it simply represents how memory is encoded. If GLR-1 expression is actually part of the memory mechanism, this would suggest that for habituation, the molecular mechanisms underlying consolidation are the same as mechanisms of reconsolidation. Because a number of studies have demonstrated that both consolidation and reconsolidation rely on protein synthesis (e.g., Nader et al., 2000, Sangha et al., 2003c) it has been hypothesized that reconsolidation is simply a recapitulation of the molecular process thought to underlie consolidation. However, Przybyslawski, et al. (1999) and Anokhin et al. (2002) both found that a lower dose of a pharmacological blocker administered after memory reactivation abolished memory than was required if administered during training suggesting there is a different level of blockade sensitivity between consolidation and reconsolidation periods. Other studies have reported that consolidation and reconsolidation produce differential effects to the
same manipulation. For instance, Taubenfeld et al. (2001) found that consolidation of an inhibitory avoidance task in rats relied on hippocampal C/EBPβ (an immediate early gene activated by CREB) while reconsolidation of this task did not. As well, Eisenberg and Dudai (2004) found in Medaka (goldfish) that memory reinstatement (exposure to the unconditioned stimulus-alone) was only effective at reinstating memory that had previously been abolished by blockade of reconsolidation; they found no reinstatement for memory when consolidation was blocked. As well, von Hertzen and Giese (2005) found memory consolidation and reconsolidation result in an upregulation of one immediate early gene (SGK-3), however memory consolidation alone resulted in the upregulation of another immediate early gene (NGFI-B) providing evidence for both hypotheses of mechanisms of memory reconsolidation; reconsolidation is a recapitulation of consolidation versus reconsolidation and consolidation rely on distinct mechanisms. Although I have shown evidence that memory consolidation and reconsolidation both produce similar changes in GLR-1 expression, it is possible that memory consolidation and memory reconsolidation activate different molecular cascades that both serve to redistribute GLR-1-containing receptors (i.e., AMPA-type glutamate receptors) thus allowing for disparate molecular mechanisms, one for consolidation and another for reconsolidation, to result in a similar change in GLR-1 expression. Further experimentation is required to determine the nature of the relationship between consolidation and reconsolidation.

Another question that must be addressed is whether the memory is actually abolished or whether expression of memory is merely compromised. Inhibiting reconsolidation seems to selectively affect newly established memories while longer
retained memories seem to be resilient (see Dudai, 2004 for review). Milekic and Alberini (2002) demonstrated using inhibitory avoidance in the rat, that inhibition of reconsolidation produced a memory deficit for more recently learned avoidance memory, but not if learning occurred 2 weeks prior to reconsolidation inhibition suggesting that older established memories are more resilient to reconsolidation inhibition. These reports suggest that reconsolidation of more recently formed memories may rely on the same cellular mechanism while reconsolidation of a long-established memory may rely on a different mechanism altogether. As these experiments were conducted in rats, another possibility is that long-established memories may go through another, later occurring process that somehow makes them more impenetrable to disruption. Further investigation into the permanence of long-term memory for habituation in C. elegans will be an important step in investigating this. For example, if reconsolidation blockade completely erases the memory for habituation then rehabituation should occur at the same rate as for naïve worms. In contrast, if reconsolidation blockade has not erased the complete memory for habituation there would be more rapid rehabituation in worms that underwent reconsolidation blockade than in naïve worms.

These data represent the first demonstration that the cellular correlate of a memory (in this case a decrease in GLR-1 expression) is eliminated when reconsolidation is blocked. The results reported here support the hypothesis that reconsolidation requires a similar mechanism of plasticity as consolidation. In addition, this is the first demonstration that memory for non-associative learning shows a similar level of plasticity following reactivation as has been seen with memory for associative learning.
suggesting a basic biological process underlies all forms of memory induced synaptic modification.
CHAPTER 5

Objective 4: Genetically Dissociable Memory Phases in Caenorhabditis elegans

5.1 INTRODUCTION

Memory research using animal models has led to the understanding that memory at different time points after training can be distinguished by different molecular mechanisms underlying the behavioral expression of memory. Further, different forms of memory have been shown to be produced by particular training protocols (i.e., massed vs. spaced), the amount of stimulation (Sutton et al., 2000) and the temporal pattern of stimulus presentation (Beck and Rankin, 1997). It is apparent from this research that nervous systems use the temporal features of experience to store memory and that the type of memory that is formed will depend on the temporal features of the stimulation the organism experiences.

Research from several organisms has shown that duration of memory for a learned experience depends on the temporal parameters employed during training. As discussed in Chapter One, studies in Drosophila examining long-term memory for an odor avoidance task can be produced by repeated conditioning trials spaced over time (10 trials, one every 15 minutes). Massed training (10 trials, one immediately after another) produces a different lasting memory phase in Drosophila termed anaesthesia-resistant memory (ARM) thought to occur simultaneously with long-term memory (Tully et al. 1994). Both forms of memory share distinct characteristics with regards to training protocols required to produce the memory and protein synthesis dependence; ARM occurs independently of protein synthesis. As well, ARM lasts up to 4 days while LTM
lasts at least 7 days. Similarly, in *Aplysia*, long-term retention (>24 hours) for sensitization has been shown to occur in at least two lasting forms; a long-term phase lasting >24 hours and an intermediate phase lasting ~ 90 minutes (Sutton et al., 2000). Unlike in *Drosophila*, these two lasting forms of memory for sensitization in *Aplysia* both rely on protein synthesis even though intermediate-term memory decays before there is evidence of long-term memory. Long-term memory for habituation in *Aplysia* has similar characteristics to long-term memory in other systems; namely, it requires distributed training and protein synthesis. As well, lasting memory for habituation (>6 hours) has been shown to require post-synaptic glutamate receptor activation and protein synthesis (Ezzedine and Glanzman, 2003).

In *C. elegans*, long-term memory for habituation in *C. elegans* has been shown to require a distributed (spaced) training protocol (Beck and Rankin, 1997; see Chapter Two), protein synthesis (Beck and Rankin, 1995; see Chapter Two) and intact glutamate transmission (Rose et al., 2002; see Chapter Three). Further, this memory has also been shown to require post-synaptic non-NMDA-type glutamate receptor activation and to produce a significant decrease in GLR-1 with no corresponding change in presynaptic synaptobrevin (vesicle associated protein) expression (Rose et al., 2003; see Chapter Three).

The current study was designed to investigate whether memory for habituation would be seen 12 hours after either distributed or massed training. I found that memory for habituation could be measured at ~12 hours following both massed and distributed training. As well, I examined whether this early memory relied on glutamate transmission similar to the long-term memory reported in Chapter Three by examining
the same mutant strains reported in Chapter Three that did not show memory at 24 hours following distributed training (i.e., eat-4 and glr-1; Rose et al., 2002; Rose et al., 2003). Using the GFP transgenic strains described in Chapter Three, I also examined whether memory at 12 hours is correlated with a similar decrease in GLR-1 as was seen in 24 hour memory or whether it produces any change in synaptobrevin expression. These GFP studies led to the hypothesis that the memory seen 12 hours after distributed training was different from the memory seen 12 hours after massed training. Finally, I uncovered a putative mechanism for memory seen 12 hours following massed training. These studies demonstrate the presence of multiple mechanisms of memory in *C. elegans*.

### 5.2 METHODS

**Subjects**

A total of 183 hermaphroditic wild-type *C. elegans* Bristol (N2), 90 eat-4(ky5), 114 glr-1(n2461), 32 GLR-1::GFP, 72 pmec-7::SNB-1::GFP and 98 flp-4::flp-8::flp-20 worms were used. N2 and glr-1(n2461) worms were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). The glr-1(n2461) gene has been reported to code for a non-NMDA-type glutamate receptor that is 40% homologous to mammalian AMPA-type glutamate receptors (Hart et al., 1995). eat-4 mutant worms were originally obtained from the laboratory of Dr. Leon Avery at the University of Texas Medical School. eat-4(ky5) is a loss-of-function, putative null allele of a glutamate vesicular transporter (Avery 1993). Lee et al. (1999) showed that eat-4 gene activity was localized to a 6.9-kb region of cosmid ZK512. GLR-1::GFP was from A. Hart (Harvard University, Boston, MA), and pmec-7::SNB-1::GFP was from M. Nonet (Washington
University, St. Louis, MO). Finally, the \textit{flp-4;flp-8;flp-20} was obtained from Chris Li (City University of New York, New York, NY). All worms were maintained on Nematode Growth Medium (NGM) agar seeded with \textit{Escherichia coli} (OP50) as described in Brenner (1974).

\textit{Behavioral Testing}

Habituation training and testing took place under the same conditions as the previous chapters (See Chapter Two and Three). Again, training was not recorded;

\textbf{Figure 16. Massed and Distributed Training Protocol for 12-Hour Retention.} Worms were trained in groups with either a single block of 80 taps (massed training) or 4 blocks of 20 taps separated by 1-hour rest periods (distributed training). Both trained and control worms are transferred to individual plates one hour after training and then undergo a 12-hour delay interval. Testing consists of both control and trained worms receiving 10 taps and their responses to these 10 taps are measured.
therefore the training phase occurred without the microscope stage illuminated. Testing occurred with the stage light on and was video-recorded. Habituation stimuli were administered via electrical stimulation from a Grass S88 stimulator resulting in a mechanical tapper exerting 1–2 N of force to the side of the petri plate on which worms were kept thus producing a single tap stimulus.

The distributed training protocol consisted of an experimental group of ~20 4-day-old worms trained simultaneously on a single plate with four blocks of training (see figure 16). Each training block consisted of 20 tap stimuli given at a 60-sec ISI with 1-hour rest periods between blocks. A control group that included another 15–20 worms received only a single tap on training day. Multiple experimental and control group were included in each experiment. At least 1 hour after the last tap of training, worms from both the trained and control plates were transferred onto labeled, individual *E. coli*-streaked plates. Worms from both groups were tested one at a time with 10 single taps given at a 60-sec ISI: testing occurred 22–28 h following training (median >24 h). Memory was measured as a comparison of average response magnitude to the 10 test single taps between the trained group and the untrained control group. Long-term memory for habituation was measured as a significant difference using a between-groups comparison for the average test responses between the experimental and control groups.

The same procedures were employed for massed training, except that habituation training was delivered in a single training block (80 taps delivered at a 60s ISI). So that the worms tested 12 hours after training were the same age as worms tested 24 hours after training, 4 ½ day-old worms were trained and then tested 10-15 hours later at 5 days of
age. All other aspects of training and testing were performed in the same manner as in the distributed long-term memory protocol (see above).

**Scoring**

Testing was recorded for all worms and scored with the same methods as described in Chapter Two.

**Heat Shock Treatment**

Heat shock was administered in the same fashion as described in Chapter Two.

**Imaging**

GFP transgenic worm strains were mounted onto welled slides using 12-15 µl of 2,3-butanedione monoxime for paralysis mixed with medium diameter Sephadex beads to prevent the slide cover from crushing the worms. Fluorescence imaging of the GFP transgenic strains used the same equipment described in Chapter Four. Again, in all cases, a SETCOL look-up table was used during image collection with gain and offset set to allow for imaging at an optimal intensity. Twelve hours prior to imaging, GLR-1::GFP and pmec-7::SNB-1::GFP worms were given either massed or distributed training; each trained group was paired with an untrained control group. The GLR-1::GFP strain expressed GFP along the ventral cord, and images were collected along the posterior portion from the tail to the vulva (~3 stacks/worm). Images of GLR-1::GFP expression were composed of 12-20 optical sections. A researcher blind to the treatment groups coded image files to allow for blind measuring of images. Using Image J, collected
images were stacked, thresholded and measured. Total GFP expression was measured as the area of GFP expression (microns squared). Number of GFP expressing clusters was tallies in Image J using the Count function.

Twelve hours prior to imaging, $\rho$mec-7::SNB-1::GFP worms underwent either massed or distributed training or served as an untrained control. On test day, images were collected using the same mounting and imaging method as detailed above. GFP expressed in the $\rho$mec-7::SNB-1::GFP worms was captured in a single stack composed of ~16-30 optical sections. Since we examine the same 2-3 GFP clusters reported to be extending from PLM (Nonet, 1999), we only calculated total GFP expression (microns squared). Final figures were generated using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Data Analyses

All data analyses were conducted using Statview 4.5. All reversal magnitude values were averaged across testing. ANOVA’s were run on all data with the Newman-Keuls test for post-hoc analyses. In graphs, responses are expressed as percent control response (responses are divided by mean control response and multiplied by 100).

5.3 RESULTS

Experiment 5A. Massed Training Produces Early Retention

In Chapter Three I reported that distributed habituation training produced long-term retention that lasted at least 24 hours (Rose et al. 2002; Rose et al., 2003). In this experiment I investigated whether there was 12-hour retention following either a
distributed or a massed training protocol (see figure 16). Although distributed training with taps delivered at a short ISI results in no memory 24 hours after training, I investigated whether retention could be seen 12 hours after training with a short ISI. In this investigation I looked for 12-hour memory in three conditions: following distributed training with a 60 s ISI, following massed training with a 60 s ISI, and following distributed training with a 10 s ISI. In each condition there was a trained group and an untrained control group. An overall ANOVA showed significant differences between groups (F(5,102)=14.334, p<0.001; figure 17). Post-hoc analyses show a significant difference in responding between control and trained worms that received distributed training at a 60s ISI (p=0.006). Massed training at a 60s ISI also produced significantly smaller responses in trained worms compared to controls (p=0.013), however, worms

![Graph showing mean reversal magnitudes of worms](image.png)

**Figure 17. Massed training produces retention at 12 hours after training.** Mean reversal magnitudes (±SEM) of worms that received training 12 hours earlier compared to controls that received a single tap on training day. Worms that received distributed training with taps delivered at a long ISI (Long Distributed) showed retention at 12 hours after training, as did worms that received massed training with taps delivered at a long ISI (Long Massed); distributed training at a short ISI (Short Distributed) did not result in memory. *=p<0.05.
given distributed training at a 10s ISI showed no difference in level of responding compared to controls at 12 hours after training (p=0.837). Because distributed training is required for 24-hour memory, it was not surprising to find that distributed training also results in memory for habituation 12 hours after training. However, this experiment also indicated that massed training produces a form of memory that is measurable 12 hours after training. This memory 12 hours following massed training is likely temporary in nature as it has previously been shown that massed training does not produce retention at 24 hours, therefore, the memory reported here 12 hours after massed training is no longer measurable 24 hours after training.

Experiment 5B. Memory at 12 hours Post-Training is Insensitive to Protein Synthesis Inhibition

My next goal was to determine whether the memory that was observed 12 hours after massed or distributed training relied on the synthesis of new proteins as was shown for 24-hour memory following distributed training (see Chapter Two).

To examine the effects of inhibiting protein synthesis on 12-hour memory following massed training, worms were subjected to heat shock following the single massed training block. A no shock condition served as a control. An overall ANOVA was significant (F(3,79) = 3.917, p=0.012; see Figure 18). Post-hoc analyses revealed that worms that received massed training show significant 12-hour retention whether no shock followed training (p=0.034) or heat shock was delivered after training (p=0.009). This suggests that memory 12 hours following a massed training protocol does not rely on protein synthesis.
To examine whether 12-hour memory following distributed training was protein synthesis dependent, worms were subjected to heat shock after each of the blocks of training (as described in Chapter Two).

**Figure 18. Retention at 12 hours after training does not require protein synthesis.** Mean reversal magnitudes (±SEM) 12 hours after training. A) wild-type worms that received massed training followed immediately with heat shock for 40 minutes still show memory at 12 hours after training. B) wild-type worms that received distributed training with heat shock delivered for 40 minutes after each training block also show 12-hour memory. *p<0.05.

An overall ANOVA on trained and control worms receiving heat shock or no shock showed significant differences (F(3, 86)=4.207, p=0.008 see figure 18). Post-hoc analyses indicated that trained worms responded with significantly smaller responses than controls in both the no shock control condition (p=0.013) and the heat shock condition (p=0.019), indicating that memory 12 hours after distributed training was not disrupted by heat shock. From these two experiments it appears that memory 12 hours after training, does not require protein synthesis regardless of the training protocol.

*Experiment 5C. Glutamate Transmission is Not Required for 12-hour Retention*
The memory that is seen 24 hours following distributed training relies on intact glutamate transmission (Chapter Three). I found that worm strains carrying mutations that interfere with either the availability of glutamate at the chemical synapses in the tap-withdrawal response circuit (eat-4; a glutamate vesicular transporter; Lee et al., 1999) or mutation of glutamate receptors on the interneurons within this same circuit (glr-1; homolog of mammalian AMPA-type glutamate receptors; Hart et al., 1995) also interfere with long-term memory for habituation. That is, neither eat-4 worms nor glr-1 worms show memory 24 hours after distributed training (Chapter Three). In the next experiment I tested whether the memory seen 12 hours after massed training or after distributed training required intact glutamate transmission and whether inhibition of protein synthesis affects any memory expressed by the mutant strains 12 hours after training.

In the first experiment I tested the eat-4 mutant strain 12 hours after massed training in both a no shock or heat shock condition. An overall ANOVA revealed significance (F(3, 47)=17.210, p<0.001; see figure 19a). Post-hoc comparisons showed that the trained worms responded with smaller reversals than control eat-4 mutant worms indicating memory was there. As well, if heat shock was delivered after training, trained eat-4 mutant worms still produced significantly smaller reversals than eat-4 controls (p=0.020). These data show that proper eat-4 gene functioning was not required for 12-hour memory following massed training. Further, the memory that was seen with the eat-4 mutant strain 12 hours after massed training did not rely on protein synthesis as heat shock delivered immediately after training had no effect on 12-hour retention following massed training.
In the second experiment I examined 12 hour memory following massed training in one of the glr-1 mutant strains (glr-1(n2461); Hart et al., 1995) that failed to show memory 24 hours after distributed training (Chapter Three). Similarly, I also examined whether heat shock delivered after training had an effect on 12-hour memory following massed training in the glr-1 mutant strain. An overall ANOVA revealed a significant difference (F(3, 69)=10.740, p<0.001; see figure 19b). Post-hoc analyses showed that

![Figure 19](image_url)

**Figure 19. Twelve-hour retention is not glutamate dependent.** A) Mean reversal magnitudes (±SEM) at testing, eat-4 mutant worms still show 12-hour retention after massed training that is unaffected by protein synthesis inhibition, B) Mean reversal magnitudes (±SEM) at testing, glr-1 mutant worms show retained 12-hour memory after massed training that is unaffected by protein synthesis inhibition, C) Mean reversal magnitudes (±SEM) at testing, eat-4 and glr-1 mutant worms both show 12-hour retention after distributed training. * = p<0.05.
glr-1 mutant worms that received massed training show significantly smaller responses 12 hours after training compared to glr-1 controls (p=0.005) and that heat shock delivered immediately after massed training had no effect on this difference (p=0.043). These results are similar to what was seen with the eat-4 mutant strain; memory was present in glr-1 worms 12 hours after massed training and this memory was also resistant protein synthesis inhibition by heat shock. Taken together these data suggest that memory 12 hours following massed training does not rely on glutamate transmission. Further, it demonstrates that even with limited glutamate neurotransmission, the remaining memory is not dependent upon protein synthesis.

The data thus far showed that memory 24 hours after distributed training requires intact glutamate transmission (Rose et al. 2002; Rose et al., 2003), while memory 12 hours after massed training does not. This raised the question of whether memory 12 hours after distributed training also requires intact glutamate transmission. To test this, I examined whether eat-4 and glr-1 were necessary for memory resulting 12 hours after a distributed training protocol. An overall ANOVA showed significance (F(3,76)=3.362, p=0.023; see figure 19c) and post-hoc analyses revealed that trained worms responded with significantly smaller reversal compared to strain-matched controls for both the eat-4 mutant strain (p=0.024) and the glr-1 mutant strain (p=0.039). This was an unexpected result as memory at 24 hours post-training following a distributed training protocol does require the eat-4 and glr-1 genes. Two possible explanations could account for the discrepancy in glutamate dependence between 12-hour and 24-hour retention following distributed training; 1) these genes are perhaps only necessary for the later half of the retention interval for 24-hour memory or, 2) perhaps a second memory process is
simultaneously being recruited by distributed training and that is what is mediating the memory observed 12 hours after training. This second process must decay between 12 and 24 hours after training, as the memory seen at 24 hours is fully protein synthesis dependent.

Experiment 5D. Training Protocol Determines Changes in GLR-1 Expression

In Chapter Three, I showed that GLR-1 expression was decreased 24 hours after distributed training with no change in the number of GLR-1 expressing clusters implying that long-term memory for habituation is coded as a decrease in GLR-1 containing receptors at each synapse, but no change in the number of synapses (Rose et al., 2003). Therefore, in this series of experiments I examined whether GLR-1 shows a difference in expression 12 hours after training by using confocal fluorescence imaging of a GLR-1::GFP transgenic strain. As 12-hour retention following massed training does not appear to rely on glutamate I hypothesized that I would not see a significant difference in GLR-1::GFP expression 12 hours after training. When GLR-1::GFP worms were given massed training and then imaged 12 hours later, there was no significant change in GLR-1::GFP expression detected between control and trained worms ($t(14)=0.22$, $p=0.829$) and no change in the number of GLR-1::GFP expressing clusters ($t(14)=0.758$, $p=0.461$).

Because glutamate did not appear to play a role in 12-hour memory following distributed training but has been shown to be required for retention at 24 hours after training I examined GLR-1::GFP expression 12 hours after distributed training. If glutamate is truly only required for the latter half of the 24-hour retention interval, it would follow that I would see no difference in GLR-1::GFP expression at 12 hours post-
training. However, if distributed training activates more than one memory-associated cellular process that occur in parallel at 12 hours, including long-term memory, then a decrease in GLR-1::GFP might be noted at 12 hours following distributed training. When GLR-1::GFP transgenic worms were examined 12 hours after distributed training, a significant decrease in total GLR-1::GFP expression was seen with trained worms expressing significantly less GLR-1::GFP than control worms (t(14)=2.602, p=0.020; see figure 20 a&b). No difference in the number of GLR-1::GFP expressing clusters between control and trained worms was seen 12 hours after distributed training (t(14)=0.295, p=0.773; figure 20c). It is interesting that although the decrease in glutamate receptors was seen both 12 and 24 hours after distributed training, glutamate transmission was necessary for 24 hour memory, but not for 12 hour memory. This suggests that the decrease in glutamate receptors 12 hours after training is not necessary for the memory but that it becomes necessary by 24 hours. The implication of this is that a second

Figure 20. GLR-1 expression is still decreased at 12 hours after distributed training. A) confocal images of GLR-1::GFP expression at 12 hours after distributed training, B) quantification of GLR-1::GFP expression measured as microns squared per animal, C) tally of the number of GLR-1::GFP expressing clusters per animal. *p<0.05.
memory process is recruited by distributed training, and it is this second process that is critical for the expression of memory 12 hours after training.

Experiment 5E. Memory Following Massed Training Produces a Change in Vesicles

In my studies of memory 24 hours after distributed training (see Chapter Three) I found that although there was a down-regulation of glr-1 glutamate receptors 24 hours after training, there was no change in expression of the presynaptic vesicle protein SNB-1 (synaptobrevin; Nonet, 1999). To examine SNB-1 expression I used a SNB-1::GFP transgenic strain where the construct was behind a mec-7 promoter, therefore, restricting SNB-1::GFP expression to the presynaptic mechanosensory neurons of the tap-withdrawal circuit (Wicks and Rankin, 1997).

To investigate the role of SNB-1 in memory following massed training in the current experiments I performed confocal fluorescence imaging on pmec-7::SNB-1::GFP transgenic worms 12 and 24 hours after massed training. Twelve hours after massed training, trained pmec-7::SNB-1::GFP worms expressed significantly more SNB-1::GFP (t(36) = 2.820, p=0.008; see figure 21 a&b). When I imaged 24 hours after massed training there was no difference in SNB-1::GFP expression between control and trained worms (t(21) = 0.521, p=0.607) suggesting that the presynaptic increase in synaptobrevin is temporary and decays to baseline by 24 hours after training. Thus, these data suggest that 12-hour memory produced by massed training relies on a presynaptic mechanism that results in a temporary increase in the vesicle protein synaptobrevin.

Having found an increase in SNB-1::GFP 12 hours after massed training I examined SNB-1::GFP 12 hours following distributed training. There was no significant
change in SNB-1::GFP expression between control and trained worms 12 hours after distributed training ($t(32)=0.713$, $p=0.481$). These data, together with the observation that there is a decrease in GLR-1::GFP 12 hours following distributed but not massed training suggest that 12-hour memory following massed training is mediated by a different mechanism than 12-hour memory following distributed training.

**Experiment 5F. Memory Following Massed Training Requires FMRF-amide**

The observations that massed training produced a significant increase in the vesicle associated protein synaptobrevin, and that glutamate transmission was not necessary for memory 12 hours after massed training lead me to investigate other genes that are expressed in the presynaptic mechanosensory neurons of the tap-withdrawal circuit that might play a role in 12-hour memory following massed training. One protein that has been associated with long-term synaptic inhibition in *Aplysia*, is the neuropeptide
Phe-Met-Arg-Phe-amide (FMRF-amide; Montarolo et al., 1988). First isolated in a mollusk for its cardioactive properties (Price and Greenberg, 1977), FMRF-amide related peptides are found in most organisms in the animal kingdom. Montarolo et al. (1988) found long-term depression of synaptic transmission in cultured *Aplysia* neurons that lasted at least 24 hours following five applications of FMRF-amide. As memory for habituation likely produces a synaptic depression, I hypothesized that FMRF-amide might play a role in memory seen 12 hours following massed training.

There are at least 22 members of the *flp* family of genes (FMRF-amide-like-peptide) in *C. elegans* (Li et al., 1999). Three of the *flp* genes are expressed on the mechanosensory neurons of our circuit: *flp-4, flp-8* and *flp-20*. We tested a triple mutant strain (*flp-4;flp-8;flp-20*) for retention 12 and 24 hours following massed training and 24

![Bar graph](image)

**Figure 22. FMRFamide like peptides are required for memory following massed training.** Effect of the absence of FMRFamide in the mechanosensory neurons on 12-hour memory resulting from massed training using a *flp* triple mutant strain (*flp-4;flp-8;flp-20*), *flp-4;flp-8;flp-20* mutant worms showed no 12-hour memory after massed training (12 hr Massed), and still showed no retention at 24 hours after massed training (24 hr Massed), however, the *flp-4;flp-8;flp-20* mutant worms did show 24-hour memory following distributed training. **=*p<0.01.
hours following distributed training. An overall ANOVA showed significance (F(5, 92) = 2.888, p=0.018; see figure 22). Post-hoc analyses revealed that there was no difference in responding between control and trained flp-4;flp-8;flp-20 mutant worms 12 hours following massed training (p=0.745). We also looked 24 hours after massed training to see if this memory deficit persisted and there was no difference in responding between control and trained flp-4;flp-8;flp-20 mutant worms 24 hours after massed training (p=0.968). However, trained flp-4;flp-8;flp-20 mutant worms showed significantly smaller responses than controls 24 hours after distributed training (p=0.004). These data, together with the increase observed in SNB-1::GFP suggest that the memory seen 12 hours after massed training might be the result of increased presynaptic release of an inhibitory FMRFamide neuropeptide. Further, this proposed mechanism appears to act independently from mechanisms involved in long-term memory.

5.4 DISCUSSION

In Chapter Three I reported that long-term memory (>24 hours) requires a distributed training protocol with stimuli presented at a long interstimulus interval (60 s). The results from the current series of experiments suggest the existence of at least two additional forms of lasting memory that can be measured; one was observed 12 hours after massed training and the other 12 hours after distributed training (see Table 1). I also showed that when a shorter interstimulus interval (10 s) is employed with a distributed habituation training protocol, no memory was seen 12 hours post-training indicating that the rate at which stimuli are presented is still a necessary component for memory expressed at this time point. This finding suggests that integration of several temporal
Table 1. Summary of results following distributed and massed training. Findings are sorted by retention period (12 vs 24 hours).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>12 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Distributed Training</strong></td>
<td>Not Glutamate dependent</td>
<td>Glutamate dependent</td>
</tr>
<tr>
<td></td>
<td>Not Protein Synthesis Dependent</td>
<td>Protein Synthesis Dependent</td>
</tr>
<tr>
<td></td>
<td>Down Regulation of glr-1</td>
<td>Down Regulation of glr-1</td>
</tr>
<tr>
<td></td>
<td>No change in SNB-1::GFP</td>
<td>No change in SNB-1::GFP</td>
</tr>
<tr>
<td></td>
<td>?</td>
<td></td>
</tr>
<tr>
<td><strong>Massed Training</strong></td>
<td>Not Glutamate dependent</td>
<td>NO MEMORY</td>
</tr>
<tr>
<td></td>
<td>Not Protein Synthesis Dependent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No change in glr-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Up Regulation of SNB-1::GFP</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>flp-4,8,20</em></td>
<td></td>
</tr>
</tbody>
</table>

In contrast to 24-hour long-term memory following distributed training, memory 12 hours after distributed training does not rely on protein synthesis as heat shock administered during the training period did not block retention. One possible explanation for this is that distributed training activates several memory processes and that the memory processes that underlie 12- and 24-hour retention are simultaneously expressed 12 hours after training, thus the lack of sensitivity to protein synthesis inhibition following distributed training could be due to the expression of over-lapping yet independent forms of memory expressed at 12 hours. Memory 12 hours after massed training does not rely on protein synthesis as heat shock administered immediately after training did not block retention; memory is not seen 24 hours following massed training. This data supports the hypothesis that there are at least three phases of lasting memory in
C. elegans: massed training produces a single memory form that can be seen 12 hours after training, whereas distributed training recruits at least two forms of memory, one protein synthesis independent form seen 12 hours after training, and one protein synthesis dependent form seen 24 hours after training.

Glutamate has been shown to be important for lasting memory for habituation in both C. elegans and Aplysia (Ezzedine and Glanzman, 2003; Rose et al., 2002; Rose et al., 2003). Interestingly, in contrast to my results with memory 24 hours after training and Ezzeddine and Glanzman’s (2003) results with Aplysia, in these experiments memory was seen 12 hours following distributed training in both the eat-4 and glr-1 mutant strains suggesting that memory produced by distributed training results in either a delayed activation of glutamate-dependent processes, or again that, a second, non glutamate dependent memory mechanism was responsible for the behavioral expression of memory. Evidence in support of this latter hypothesis is provided by the finding that GLR-1::GFP expression was significantly decreased in trained worms 12 hours after distributed training; this result can be explained by the hypothesis that both glutamate-dependent and glutamate-independent memory processes are activated by distributed training and that the decrease in GLR-1::GFP expression would result from the glutamate-dependent memory process while the recruitment of another simultaneously occurring memory mechanism that does not rely on glutamate can explain the behavioral expression of memory in the eat-4 and glr-1 strains 12 hours following distributed training. This is a plausible hypothesis as a visual examination of the difference between trained and control worms following distributed training is larger than the difference between trained and control worms following massed training (Figure 17) and this is approaching significance.
(p=0.058). However, no increase in synaptobrevin::GFP was seen 12 hours after distributed training, so although it is likely that a second, non-glutamate dependent memory process is active 12 hours following distributed training, it does not appear to be the same memory mechanism that is present 12 hours following massed training.

Interestingly, these experiments show that 12-hour retention produced by massed training, is not protein synthesis dependent. Coupled with the finding that 12 hours following massed training there is also an increase in synaptobrevin GFP expression, it suggests that this increase in vesicle-associated GFP expression occurs independently of protein synthesis. One possibility is that prior to training, vesicles are distributed along the neural process and massed training results in the grouping of vesicles. This vesicle migration into dense groups would result in an increased measureable area of synaptobrevin GFP expression. Further, this hypothesis does not suggest increased synaptobrevin GFP expression is the result of an actual increase in vesicle number, therefore, an increase in vesicle-associated GFP expression could occur even when protein synthesis is inhibited.

Twelve-hour retention following massed training did not require glutamate transmission as mutations in eat-4 and glr-1 had no effect on memory at this time point indicating that this memory phase relies on a completely different cellular mechanism than memory seen 24 hours after distributed training. This lack of dependence upon glutamatergic processes was confirmed by no change in GLR-1::GFP expression 12 hours following massed training. However, a significant increase in synaptobrevin::GFP expression was found suggesting that massed training results in a transient increase in
vesicles, as this increase was seen 12 hours after massed training, but not 24 hours post-training.

To determine what might be being released from what appears to be an increased vesicle population, I investigated genes coding for FMRF-amide like peptides (flp) that are expressed in the presynaptic mechanosensory neurons. FMRF-amide has previously been implicated in both short- and long-term habituation in Aplysia (Montarolo et al., 1988). When I tested a strain of worms carrying mutations in three flp genes (flp-4; flp-8; flp-20) I found no retention 12 hours after massed training. This was not attributable to a generalized memory deficit as this triple mutant strain did show retention 24 hours after distributed training indicating that it was still capable of producing long-term memory. Further, these findings provide genetic evidence that memory produced by massed training relies on different genes and therefore a different mechanism than the memory produced by distributed training.

The findings reported here provide support for the hypothesis that a number of independent phases of retention exist over the entire time course of a memory. This might mean that a single experience is serially encoded into various memory phases over time. This may seem overly redundant, however, it could be that the purpose of some memory phases is to preserve the memory trace until a more permanent memory process has successfully encoded the memory for storage. If this were true, then deficits in lasting memory should occur as a result of inhibition of short-term memory processes. However, studies have shown long-term memory can occur in the absence of short-term memory (Izquierdo et al., 1998; Vianna et al., 1999), or long-term changes in the molecular correlate of memory (change in neural communication) in the absence of short-term
changes (Emptage and Carew, 1993). Thus, it seems that the abolition of at least some forms of short-term memory do not effect the formation of long-term memory. Recently, Isabel et al. (2004) reported that the two lasting memory phases in Drosophila (ARM and LTM) not only occur in parallel, but are completely independent forms of memory lending support to the notion that several memory processes may be initiated after a learning event. This suggests the hypothesis that some of the processes may be serial, and some may occur in a parallel fashion.

These findings demonstrate that memory can exist in several forms that can be dissociated genetically and thus mechanistically. I found that memory at 12 hours following a massed training protocol relies on a completely different family of genes (ftp’s) compared to 24-hour long-term memory (eat-4 and glr-1). Further, a distributed training protocol appears to activate several memory phases simultaneously; one that mediates 24-hour long-term memory that is dependent on glutamate associated genes and, another glutamate-independent memory process that mediates memory at 12 hours. These findings will help elucidate the nature and mechanism of memory. Evidence from this study supports the importance of temporal features of experience for memory encoding, and that duration of retention depends, at least in part, on the temporal organization of the training protocol.
CHAPTER 6

6.1 DISCUSSION

LONG-TERM MEMORY REQUIRES GENE TRANSCRIPTION

The objective of the research described in this dissertation was to further the understanding of memory mechanisms using *C. elegans* as a model system. To study memory I first began by establishing a new training protocol to produce memory and then demonstrated the importance of protein synthesis and glutamate transmission in this memory. Through these studies I also determined that *glr-1* expression changes as a result of long-term memory. I then demonstrated that, at least under certain conditions, long-term memory is modifiable at later time points bringing into question the permanence of memory and confirming that long-term memory for habituation depends on subsequent reconsolidation into a stable state once reactivated from memory stores. Finally, I investigated the conditions required to show memory at a shorter retention interval and uncovered two different forms of memory and determined a putative mechanism for one of these forms of memory that involves the neuropeptide FMRFamide.

In Chapter One, I successfully replicated earlier findings (Beck and Rankin, 1995; 1997) in long-term memory for habituation in *C. elegans*. Previously, using a time-consuming within-subjects training protocol, it had been found that long-term memory required training be administered using a distributed or spaced training protocol; no long-term memory was seen if the same number of habituating stimuli were presented in a
single training block (Beck and Rankin, 1997). From a cognitive perspective, this ‘spacing effect’ has been said to occur because when training occurs at different points across time, it is likely that more information and thus more associations with the various training environments are made. This greater number of associations is thought to allow for enhanced recall at a later time (Greene, 1989). Further, it has been shown with mice that the spacing effect may preferentially improve retention performance for environmental cues. Scharf et al. (2001) found using fear conditioning (associating a tone with shock) in mice that spaced training enhanced long-term memory for contextual but not cued fear conditioning. (Scharf et al., 2001). This would follow if during each training trial the animal is remembering more and more environmental cues to allowing the animal to predict the occurrence of a shock at some later time. Interestingly, massed training did not have an enhancing effect on cued fear conditioning (where shock follows a discrete tone). Thus it is possible that spacing training over time allows for more associations to be made with the learning environment and thus enhances long-term memory recall.

However, from a biological perspective, it is theorized that distributed training results in enhanced retention because each training block triggers a molecular cascade responsible for the biological coding of memory. For instance, it has been reported that long-term memory results in long-lasting cellular changes (see Chapter 3 and Bailey & Chen, 1983; 1988) which require gene transcription. It has been established that activation of the transcription factor CREB requires an increase in intracellular calcium (Abel et al., 1997). This increase in calcium concentration was traditionally thought to occur from calcium influx into the cell through receptor-associated ion channels, in
particular, glutamate-receptor-associated channels. Abel et al. (1997) demonstrated that although a single training trial did produce an increase in calcium, it was not sufficient to activate the gene transcription factor CREB. As such, any changes were temporary and no lasting changes were presumed to occur.

One explanation for my findings that distributed training results in greater retention as an increased number of training blocks are administered is that training over time allows for more memory associations. Although short-term habituation is non-associative, in terms of long-term memory habituation can be associated with context. For instance, Hermitte et al. (1999) showed in the crab Chagmanthus that greater retention results when the context during training and testing are the same. However, in the training and testing conditions I employed the contexts differed (worms underwent training in groups on an unilluminated Petri plate while testing occurred in isolated conditions in illuminated conditions; see methods Chapter Two) thus reducing the number of contextual associations. Therefore, in this case, distributed training could serve to enhance long-term memory formation by increasing the number of consolidation periods.

Interestingly, the degree of memory following three blocks versus four blocks of training did not differ significantly, although it did appear that memory after four blocks was subject to less variability and thus produced more reliable long-term memory. This correlates with what has been shown biologically in that once a certain concentration of intracellular calcium occurs, this activates a protein kinase cascade that results in the activation of gene transcription factors. Further, the properties of one particular protein kinase essential for this cascade (protein kinase A) is that once activated (phosphorylated)
it will activate itself (autophosphorylate; Abel et al., 1997). Once autophosphorylation of PKA occurs, CREB activation follows and genes are transcribed that will likely result in permanent changes in the cell to encode memory.

Additional support for the hypothesis that memory formation requires a sustained increase in Ca\(^{2+}\) for autophosphorylation of PKA to occur, is our finding that training at a short interstimulus interval (ISI) did not produce long-term memory; the repeated rapid activation presumed to occur with a short ISI, perhaps results in a pattern of change of calcium currents that is insufficient to activate gene transcription factors. Thus, no long-term memory is seen.

The postulate that long-term memory requires protein synthesis is supported by the finding that heat shock delivered during the rest periods between training blocks of a distributed training protocol abolishes long-term memory. The fact that heat shock delivered for the first 40 minutes of each rest period is sufficient to abolish long-term memory suggests that the protein synthesis that occurs within the first 40 minutes after each training block is sufficient to produce long-term memory. Interestingly, when we shortened the rest period duration from one hour to 40 minutes, this did not produce long-term memory. Thus, the one hour period of time immediately following each training block is likely the time when the molecular machinery for consolidation of memory occurs and that at least 60 minutes is required for this consolidation process to take place.

**LONG-TERM MEMORY IS GLUTAMATE-DEPENDENT**

In Chapter Two, I showed that the new between-groups training protocol is also sufficient for examining the role of specific genes in long-term memory. I began with the
eat-4 mutant strain and found that when I employed the original within-groups training protocol, I produced long-term memory for habituation, however, when I employed the new between-groups training protocol, I saw no long-term memory (see Chapter 3). Because the original training protocol used a “train of taps” (6 taps delivered in a 1 second period) as the habituating stimulus, I hypothesized that this could explain the discrepancy between the results from the two training protocols. Indeed, when trains of taps were used with the new between-group training protocol, I was able to replicate what was found with the original within-groups training protocol; eat-4 showed long-term memory for habituation.

Why would trains of taps produce long-term memory when single taps did not? Although the eat-4 gene carries a mutation for the primary glutamate vesicular transporter (putting glutamate into the vesicles for release upon stimulation), there are other glutamate transporters (e.g., Radice and Lustigman, 1996) and these may remain intact in the eat-4 mutant strain. It could follow then that the stronger train of taps stimulus could produce enough stimulation to drive the function of an alternate glutamate transporter and result in higher levels of glutamate being moved into vesicles. This idea is supported by a previous study that demonstrated no difference in the initial rate of habituation between eat-4 and wild-type worms, but the eat-4 mutant strain did show enhanced overall short-term habituation to the train of taps stimulus suggesting that the eat-4 mutant strain had glutamate available for release initially but was somehow incapable of sustained glutamate release resulting in the rapid increase in habituation. In the mammalian nervous system, glutamate transporters (or excitatory amino acid transporters) are responsible for determining the amplitude and kinetics that result from
vesicular release of glutamate (Shen and Linden, 2005). If the eat-4 mutant strain is incapable of sustained glutamate release, this would suggest that actions of glutamate are required for a certain period of time for long-term memory formation.

Once the data from eat-4 worms implicated glutamate transmission in the formation of long-term memory, my next step was to determine how sustained glutamate may be acting to produce long-term memory. I tested several glutamate receptor mutant strains (see Appendix) and found that the glr-1 mutant strain consistently showed a long-term memory deficit after distributed habituation training. I determined that the glr-1 mutant strain was capable of short-term habituation. Using two different glr-1 mutant strains I was able to confirm that glr-1 was necessary for long-term memory. Training wild-type worms in the presence of the non-NMDA-type glutamate receptor blocker, DNQX, verified the requirement of AMPA receptor activation for long-term memory. From these results it was evident that sustained glutamate release, likely acting on glr-1 containing receptors on the interneurons, is necessary to produce long-term memory.

Glutamate has been shown to play a role in memory for other learning paradigms in a variety of other model systems. For instance, memory for habituation to an electrical stimulus applied to the siphon of *Aplysia* (under normal conditions this stimulation produces a siphon withdrawal response) also relies on glutamate; when habituation training occurred in the presence of DNQX, this abolished memory (Ezzedine and Glanzman; 2003). As well, using a reward-related instrumental task (lever-pressing for a sugar pellet), Hernandez et al. (2005) found that blocking AMPA-type glutamate receptors with intra-accumbens injection of LY293558 during training specifically impaired memory for this task in rats. My results confirm the importance of AMPA
receptor activation in producing memory in *C. elegans* similar to its importance for memory formation in other organisms.

In the current studies I was able to go beyond behavioral experiments to elaborate on the importance of non-NMDA-type glutamate receptors in long-term memory by examining GLR-1::GFP expression 24 hours after long-term memory training. I found a significant decrease in total GLR-1 expression 24 hours after training. I hypothesized that these changes represent changes in receptor density at synapses. Using EAT-4 and SNB-1 fluorescent markers to visualize presynaptic areas and a GLR-1 fluorescent marker to indicate post-synaptic regions, Burbea *et al.* (2002) found with worms that carried both a GLR-1::GFP and an EAT-4::YFP construct (glows yellow under fluorescent light) that GLR-1 and EAT-4 were co-localized approximately ~43% of the time and worms that carried a GLR-1::GFP construct and a *vglr-1::SNB-1::YFP* were co-localized approximately ~38% of the time. This co-localization data supports the hypothesis that GLR-1::GFP expressing clusters are indicative of glutamatergic synapses. The fact that I saw no change in number of GLR-1::GFP expressing clusters in trained worms suggests that the significant decrease in total GLR-1::GFP in trained worms documented 24 hours after long-term memory training is due to less GLR-1 being expressed at individual synapses but no change in the total number of synapses. Thus, long-term memory for habituation not only requires AMPA-type glutamate receptor activation, but also results in an overall decrease in synaptic AMPA receptors.

Earlier in this dissertation, I postulated that mechanisms of memory for long-term habituation may resemble those responsible for long-term depression as habituation produces a decrease in responding while long-term depression results in a decreased
synaptic response. As well, there is some similarity in induction protocols between long-term memory for habituation and long-term depression; long-term memory for habituation requires a number of stimuli presented at a long interstimulus interval while long-term depression requires low-frequency (0.5 – 5 Hz) electrical stimulation with robust changes requiring several stimuli (e.g., 900; Dudek and Bear, 1992). As well, long-term depression requires protein phosphatase (Kirkwood and Bear, 1994). Ezzedine and Glanzman (2003) were able to block long-term memory for habituation in *Aplysia* by training in the presence of okadaic acid, a selective protein phosphatase inhibitor supporting the hypothesis that LTD may be a biological mechanism for long-term memory of habituation. Further, several researchers examining plasticity in hippocampal preparations report rapid internalization of AMPA receptors following induction of long-term depression (Lee et al., 2002; Man et al., 2000) resulting in fewer AMPA receptors being expressed at synapses. It is thought that this decrease in synaptic AMPA receptors underlies the sustained decrease in cellular responding that characterizes long-term depression. In *C. elegans*, endocytosis of glr-1 containing AMPA receptors has also been found to occur and has been proposed as a mechanism to determine synaptic strength (Burbea et al., 2002). In my studies, I show a decrease in GLR-1 expression following a behavioral memory protocol, suggesting that AMPA receptor density at synapses is part of the mechanism for long-term memory for habituation. It would be interesting to further explore the similarities between long-term memory for habituation and long-term depression by testing whether a selective protein phosphatase inhibitor would disrupt long-term memory for habituation in *C. elegans*. 
What remains to be determined is how decreases in GLR-1 could result in the expression of long-term memory for habituation. The tap-withdrawal response neural circuit (see Figure 2 in Chapter One) is comprised of two subcircuits (Wicks and Rankin, 1995); one circuit is responsible for forward locomotion while the other circuit is responsible for reversals. The mechanosensory neurons of the tap withdrawal circuit make chemical connections onto the interneurons of the opposing circuit, and it is at these connections where plasticity has been postulated to occur (Wicks and Rankin, 1997). Because I imaged only the posterior portion of the ventral cord, decreases in GLR-1 measured in these experiments are most likely at the synapses between the PLM mechanosensory neurons (that drive forward locomotion) and the interneurons of the reversal response circuit. However, the actual reversal response is mediated by electrical gap junctions between the mechanosensory and interneurons of the same subcircuit (i.e., the ALMs, PLMs and AVM mechanosensory neurons connect to their respective interneurons via gap junctions to generate reversals). One hypothesis is that the decrease in GLR-1 at the synapses between the sensory neurons and the interneurons makes the interneurons more resistant to later electrical activation by the sensory neurons. This could result in a decreased probability of excitation of these interneurons via their gap junction connections. One way to test this would be to carry out physiological recordings of these neurons. Unfortunately, electrophysiology in *C. elegans* is difficult and only a few researchers have succeeded at it (Nickell *et al.*, 2002).

An alternative hypothesis to explain how decreases in GLR-1 could result in the expression of long-term memory for habituation is that GLR-1 modifies cellular communication between the interneurons of the tap-withdrawal circuit. There is
conflicting evidence concerning this hypothesis. Burbea et al. (2002) determined that some of the command interneurons make chemical connections with each other and that at least some of these connections are glutamatergic. This allows for the possibility that some of the changes I observed might have been at interneuron to interneuron synapses. In contrast, Wicks and Rankin (1997) demonstrated that habituation to tap does not affect reversal/withdrawal responses for other stimuli. They found that worms that had previously been habituated to tap still showed normal withdrawal responses to a heat probe. The sensory neurons for heat differ from those for mechanosensation, however, the heat-sensing neurons feed into the same circuit of interneurons, therefore, since the withdrawal response to heat was unaffected by tap habituation, Wicks and Rankin hypothesized that any changes due to habituation were likely occurring at the synapses between the mechanosensory neurons and the withdrawal response interneurons and not at the level of the interneuron to interneuron synapses. To determine whether interneuron to interneuron synapses are changing it will be important to study the synapses of double labeled worms expressing pre- and post-synaptic markers in the same animals.

Finally, protein synthesis has been shown to be required for long-term memory for habituation and for most other learning paradigms. Because I showed that a decrease in GLR-1 expression results 24 hours after long-term memory training, and because this decrease in GLR-1 does not occur if heat shock is delivered after each training block, it follows that the reason I saw no behavioral evidence of long-term memory when heat shock was delivered after each training block (the consolidation period), is due to inhibition of a protein synthesis dependent process responsible for the GLR-1 decrease.
The GLR-1::GFP construct employed in these studies does not specifically mark membrane-bound GLR-1 versus intracellular GLR-1 expression. This means that the significant decrease in GLR-1 expression seen 24 hours after long-term memory training reflected a decrease in the overall amount of GLR-1 throughout the neuronal process. This suggests that the decrease in GLR-1 associated with long-term memory training is not simply due to an internalization of GLR-1 into the cytosol of the cell where it could be re-packaged and re-inserted into the membrane at a later time as has been seen in mammalian neurons; see Malinow and Malenka (2002). Rather, it means that GLR-1 proteins have likely been degraded and/or removed from the cell process altogether, and that overall expression levels are lower in trained worms. Whether the removal of GLR-1 proteins from the membrane is due to degradation occurring locally in the cell process or GLR-1 has been somehow transported back to the cell body remains to be determined. Burbea et al. (2002) demonstrated that endocytosis of GLR-1 relies on ubiquitination (a process by which proteins are inactivated by binding to ubiquitin; ubiquitin is a small molecule that signals cellular machinery to transport a protein to the proteasome for degradation). A similar mechanism has been found for mammalian cells whereby AMPA receptors are internalized and degraded by the ubiquitin-proteasome pathway (Patrick et al., 2003). Patrick et al. (2003) found that inhibiting the actions of the proteasome actually inhibited AMPA-induced internalization. It remains to be confirmed if ubiquitination of GLR-1 in C. elegans is activity-dependent. Nevertheless, this does provide a putative mechanism by which decreases in existing GLR-1 protein due to long-term memory for habituation could occur. Pilot data from our laboratory using quantitative real time RT PCR (real time – polymerase chain reaction; a technique to
measure amount of mRNA in cells at a particular time) has shown that trained worms show decreased GLR-1 mRNA (what proteins are translated from) compared to untrained worms (Holmes and Rankin, personal communication). This suggests that in addition to the removal of existing GLR-1 receptors, long-term memory training for habituation also produces a down regulation of glr-1 gene expression. Taken together, these data demonstrate the importance of intercellular signaling by glutamate in long-term memory.

LONG-TERM MEMORY IS NOT PERMANENT

Reconsolidation of a memory is hypothesized to occur following reactivation of memory by an associated cue. Nader et al. (2000) showed that inhibition of reconsolidation abolished memory. At this time, there is not a clear biological mechanism by which memory reconsolidation occurs. My studies have established that long-term memory for habituation in C. elegans is characterized by a change in GLR-1-containing receptors making this memory protocol ideal for examining cellular mechanisms of memory reconsolidation. In my studies of reconsolidation, a reminder (10 taps) was delivered 24 hours after training. I confirmed the findings of Nader et al. (2000), that memory reconsolidation depends on protein synthesis inhibition by showing that heat shock delivered after reminder was sufficient to block memory measured 24 hours after reminder. Further, I reported that if the reminder occurred in the presence of the non-NMDA-type glutamate receptor blocker DNQX, this also abolished memory measured 24 hours after reminder. In Chapter Three I reported that when protein synthesis inhibition followed each training block, I saw no decrease in GLR-1::GFP (control and trained worms expressed the approximately amount of GLR-1). When I delivered heat
shock immediately following reminder treatment, I saw no difference in GLR-1::GFP expression between trained and control worms. Because I previously reported that 24 hours after training (the time of the reminder treatment) I saw decreased GLR-1::GFP expression, I hypothesize that heat shock delivered after reminder blocked a protein synthesis dependent mechanism that is responsible for maintaining decreased GLR-1 concentration, thus resetting GLR-1::GFP back to baseline, control levels.

If GLR-1 expression is the end result of a memory mechanism, then the results of the reconsolidation study demonstrate that inhibition of a protein synthesis dependent mechanism returned the putative “memory trace” back to baseline. This resetting of GLR-1::GFP expression correlated with the lack of behavioral memory when reconsolidation was blocked by protein synthesis inhibition after the reminder. This correlation further supports the hypothesis that GLR-1 may somehow be acting as the code for long-term memory for habituation. What I can conclude from this research is that, in addition to behavioral expression of long-term memory, stable changes in GLR-1 expression are also made labile when reactivated at a time point after the initial training. This conclusion is not surprising given the observation that in mammalian neurons AMPA receptors cycle rapidly into and out of the synaptic membrane and that this cycling has been hypothesized to play a role in neural plasticity (Lüscher et al., 1999; Song et al., 1998).

It is somewhat unexpected that stable long-term memory would rely on the concentration of a protein that has been demonstrated to have such mobile properties. One possible explanation for this is that perhaps it is not GLR-1 concentration per se that is the cellular representation of memory, but rather, some stable protein that directs or
influences GLR-1 concentration at the synapse. In a 2002 review, Malinow and Malenka suggest mammalian “slot proteins” that in effect act as place holders for AMPA receptors at the synapse. A candidate protein for this function suggested by Malenka and Bear (2004), was post-synaptic density-95 or PSD-95, a membrane bound protein that has been shown to interact with another protein (stargazin) shown to escort AMPA receptors to the synapse (Chen et al., 2000). Malenka and Bear (2004) suggest that due to these properties, PSD-95 may serve as a docking site for synaptic AMPA receptors. If this hypothesis were true, it would mean that these “slot proteins” would be critical determinants of synaptic plasticity.

The context in which the possibility of these slot proteins was originally presented was as an explanation for how a constant level of transmission occurs despite the frequent AMPA receptor turnover. It is interesting to note that when reconsolidation was blocked with heat shock following a reminder in C. elegans, GLR-1::GFP expression returned to levels that were almost the same as control worms suggesting two things; first, that GLR-1 expression levels can increase/recover in neurons following a sustained decrease in concentration and, second, that somehow the cell “remembers” what its baseline synaptic concentration of GLR-1 was prior to memory induction. The possibility of slot proteins could explain our results in that perhaps although GLR-1 expression itself changed due to memory (in this case decreased), the proposed slot protein for those receptors did not change such that when GLR-1 expression became labile again with the reminder, and the memory reconsolidation processes were presumably inhibited by heat shock delivered immediately after the reminder, then GLR-1 expression increased, however, only back to baseline levels and not more. In other words, unless some process occurs to change the
number of slot proteins, then when receptor concentrations increase, the amount they increase by is dictated by the number of slot proteins acting at the membrane, thus providing a putative mechanism to account for how the baseline of GLR-1 concentration is set.

While the slot protein hypothesis offers one mechanism of maintaining "baseline" levels, a second possible hypothesis for the determination of baseline GLR-1 concentration, could be that the amount of stimulation experienced over development "sets" the strength of synapses in adulthood. This has been demonstrated recently in *C. elegans* studies in which worms raised in isolation show smaller reversal responses to tap and less GLR-1 than worms raised in typical colony conditions (Rose et al., 2005). This was hypothesized to be due to severely decreased levels of mechanosensory stimulation for worms raised in isolation which are not stimulated by conspecifics during development. Other studies from our laboratory show that if worms receive a lot of mechanosensory stimulation at particular time points during development this results in larger reversal responses to tap and more GLR-1 than unstimulated controls (Holmes and Rankin, in prep). Therefore, we know of two conditions that can determine GLR-1 concentration in adulthood and both seem to be experience-dependent processes. It may be that this activity dependent setting of baseline levels of GLR-1 is in itself a form of memory. One way to test whether this is the case is to examine whether inhibition of reconsolidation of long-term memory in worms raised in isolation returns the level of GLR-1 expression to the lower baseline of GLR-1 expression presumably set in development (rather than the higher level seen in control colony raised worms). Of course, this hypothesis does not necessarily exclude the "slot proteins" possibility in that
it could be the number of slot proteins that stimulation during development determines. Regardless, this is an exciting avenue for future research.

If memory undergoes reconsolidation in humans as well, the advantage to having long-term consolidated memories enter a labile state upon subsequent recall needs to be established. Several researchers have postulated that the function of memory reactivation and subsequent reconsolidation is to allow memory to act as a dynamic process that undergoes continual reorganization as a function of the ongoing experience of the organism (Tulving and Thomson, 1973; Przybyslawski and Sara, 1997). It would be next to impossible to test this hypothesis pharmacologically with human subjects, however, studies whereby false memories are produced under laboratory conditions have demonstrated that in fact memory can be modified at some later time after learning (Loftus et al., 1978). In this study, subjects viewed slides depicting an event (e.g., traffic scene) and are then given a description of the event that contains misleading information (e.g., stop sign is referred to as a yield sign). When subjects later recollect the event, they include the misinformation in their description. This could be explained by a memory reactivation-reconsolidation phenomenon whereby their memory for the traffic scene became plastic upon later recall. Because the scene was modified at the second viewing, it was the modified version of the traffic scene that was reconsolidated and therefore recalled at a later time (Loftus et al., 1978). This study using memory modification with humans shows the role reconsolidation may play in memory over time. The fact that human memory is subject to errors indicates that memory mechanisms do not discern what is being consolidated such that even false memories can be integrated into a
person’s memory repertoire. On a positive note, a memory reactivation-reconsolidation fashion of perpetual encoding would allow for correction of incorrect memories.

SEVERAL TYPES OF MEMORY ARE SEEN 12 HOURS AFTER TRAINING

In Chapter Five, I report on two other lasting types of memory that differ from long-term memory (>24 hours) for habituation in several important ways: firstly, memory 12 hours after training can be produced by either distributed or massed training, secondly, memory at 12 hours does not rely on protein synthesis, and thirdly, this memory does not require intact glutamate transmission. Memory 12 hours following massed training could be distinguished from memory 12 hours after distributed training by an increase in vesicles demonstrated by an increase in synaptobrevin expression following only massed training and a decrease in GLR-1 following only distributed training. Finally, I also showed that increased transmission of the neuropeptide FMRFamide could be a putative mechanism for memory at 12 hours after massed training.

Early in research on mechanisms of memory, the debate focused on whether short-term memory was an early stage of long-term memory, however, several studies have demonstrated that long-term memory can be produced in the absence of short-term memory (Izquierdo et al., 2002; Vianna et al., 1999) suggesting that different types of memory for some learning paradigms are produced in parallel, relying on disparate mechanisms with each memory type comprised of its own particular properties. For memory for habituation to tap we demonstrate that 12 hours following distributed training, overall retention is likely the integration of at least two or more types of memory all expressed at this time point. I provided evidence for this hypothesis when I tested the
eat-4 and glr-1 mutant strains for 12-hour retention following distributed training. Since distributed training results in long-term memory 24 hours after training, presumably the memory or at least some component of memory seen 12 hours after distributed training is long-term memory (see Table 1).

When memory was observed 12 hours following massed training the simple explanation would be that it was mediated by the same mechanism as memory 12 hours after distributed training. However, massed training did not result in a decrease in GLR-1::GFP expression like 12-hour memory following distributed training. In contrast, 12-hour memory resulting from massed training produced a significant increase in synaptobrevin expression measured by fluorescence imaging of the mec-7::SNB-1::GFP transgenic strain. This suggests that there was a presynaptic increase in vesicles associated with 12-hour memory produced by massed training, that was not seen after distributed training. The neuropeptide FMRFamide has previously been implicated in studies of long-term depression (Montarolo, et al., 1988). Li et al. (1999) reported that three flp (FMRFamide like peptides) genes are expressed in the mechanosensory neurons in the tap-withdrawal circuit. When I tested a mutant strain that carried mutations in all three flp (FMRFamide like peptides) genes expressed in the tap withdrawal neural circuit (flp-4;flp-8;flp-20) the flp triple mutant strain showed no retention 12 hours following massed training but did show 24-hour retention following distributed training. These results support the hypothesis that the genes and thus mechanisms upon which long-term memory relies are distinct from the mechanisms responsible for memory at 12 hours following massed training.
These results are the first demonstration of a complete genetic dissociation between types of memory. Studies in *Drosophila* have examined genes involved in retention for an associative odor-avoidance task and found several genes whose expression is responsible for retention at different time points (see DeZazzo and Tully, 1995). Tully *et al.* (1994) reported a similar early lasting type of memory that could also be produced by massed training, termed anesthesia-resistant memory (ARM). Similar to 12-hour memory resulting from massed training in *C. elegans*, ARM decayed earlier than long-term memory, occurs independent of protein synthesis and relies on a gene (the *radish* gene mutation was said to selectively inhibit this memory). However, flies carrying the *radish* mutation only showed a partial memory deficit and it was purported that the remaining memory was due to preserved long-term memory. Thus researchers concluded that long-term memory and ARM are distinct memory processes that act in parallel; the occurrence of one does not rely on the occurrence of the other type of memory. The results I observed in *C. elegans* differ from *Drosophila* in that the *flp* triple mutant strain showed no retention at the earlier lasting memory stage, there was no underlying long-term memory component expressed. This could have been because 24-hour long-term memory formation in *C. elegans* relies exclusively on distributed training, while in *Drosophila* lasting memory can be produced by both massed and distributed training.

It has recently been reported that memory resulting from spaced or distributed training (LTM) acts to suppress lasting memory formed from massed training (ARM); Isabel *et al.*, 2004). This was found by examining memory for spaced training in flies that were missing the α-lobe of the mushroom bodies, an area of the fly brain where LTM was
thought to be encoded. When these α-lobe absent flies were given distributed training and then tested for memory at a time point where ARM memory alone is thought to exist (~5 hours), Isabel et al. (2004) found that memory performance deteriorated. In other words, the more the flies were trained, the poorer their memory became at later retention trials. They interpreted this result to mean that distributed training not only produces LTM but also counteracts the formation of ARM. This suggests that ARM and LTM are in fact exclusively distinct forms of memory and the formation of one may influence the formation of the other. This is further evidence that several forms of memory likely occur after a learning event, and that retention can be determined by the temporal features of training.

The data presented in Chapter Five presents a clear genetic dissociation between 24-hour long-term memory following distributed training, 12 hour memory following distributed training and 12-hour memory following massed training; long-term memory relies on genes required for glutamate transmission (*eat-4* and *glr-1*), 12 hour memory following distributed training does not rely on genes for glutamate transmission and does not rely on protein synthesis while 12-hour massed training memory requires an increase in release of the neuropeptide FMRFamide. The data presented here regarding memory in *C. elegans* is the first example of a genetic dissociation between types of memory whereby in the absence of proper gene function, there is a corresponding absence of memory.

CONCLUSIONS
The experiments presented in this dissertation contribute greatly to our understanding of properties of memory and the mechanisms that underlie memory. I have established the conditions that are required for long-term memory formation in *C. elegans* and have investigated the permanence of this established, consolidated memory. I have demonstrated several genetically dissociable forms of memory that are the result of different training protocols, or different test times after training. I have also elucidated several cellular mechanisms that correlate with these forms of memory (i.e., decreased glutamate receptor expression and increased release of an inhibitory neuropeptide).

There are a few limitations to interpretations about memory that stem from my studies. Firstly, I have examined memory for a single task. There may be differences between memory for a non-associative task like the one used in these studies (i.e., habituation) and memory for associative learning paradigms. As well, I have examined memory in only a single animal model; there may be species differences in how memory is encoded and molecules employed for that encoding. Memory could be argued to be a dynamic process and these studies only examine memory at particular time points (12 and 24 hours after training). It remains to be determined what is happening at other times after training. Finally, these studies have focused on a particular subset of proteins (glutamate receptors and vesicles) and there are likely several other proteins and molecules involved in memory. Although limitations exist, these results give a starting point for examining the generality/universality of memory processes. It is reassuring to note that there are similarities between memory for habituation in *C. elegans* and memory for other tasks in other model systems.
The results of these experiments (summarized in figure 23) show that mechanisms of memory appear to be conserved across several species. The requirement of distributed training for long-term memory formation has been shown in organisms ranging from protozoa to man. The importance of protein synthesis for memory formation found in most other model systems was also seen in *C. elegans*. Interestingly, I have reported

**Figure 23. Lasting Memory Phases in *C. elegans***. Illustration of proposed types of memory that occur at respective time points following training. Each memory type relies on distinct mechanisms, yet together, these memory phases account for lasting behavioral retention in *C. elegans*. 
evidence than long-term memory formation in *C. elegans* resemble mechanisms of long-term synaptic plasticity in mammalian cells; a decrease in GLR-1 in *C. elegans* following long-term habituation training is similar to the decrease in GluR1 following the induction of long-term depression in mammalian cells. This suggests the hypothesis that glutamate receptor trafficking may be a primitive mechanism of plasticity that is conserved across phylogeny. Long-term memory for habituation in *C. elegans* was also found to be sensitive to disruption when reconsolidation was prevented. Again, this finding of reconsolidation blockade in *C. elegans* suggests that this too is a fundamental feature of memory that may exist in all organisms. Finally, the 12-hour memory phase in *C. elegans* shares characteristics (produced by massed training and does not rely on protein synthesis) seen with ARM in *Drosophila*. That we can show long-term memory in the absence of 12-hour memory (in the *flp* mutant strain) is similar to what is seen with intermediate-term memory in *Aplysia*. From this it is clear that there are many different mechanisms that can underlie memory and that many of these mechanisms are conserved across phylogeny. Together the data in this dissertation support the notion that the temporal features of an experience are the critical determinants of how the memory for a given experience is encoded. My research has shown that memory expression and the mechanisms underlying memory in *C. elegans* share many features with what we know about memory in mammalian systems. By using a model system simpler than mammals, I have contributed new insights into the fundamental characteristics of memory. Through understanding how different cellular mechanisms are recruited under different conditions and timing parameters in *C. elegans* we can move closer to understanding how memories are induced and maintained in all organisms.
FOOTNOTES

Experiments in sections 2.1-2.4 were published as: A NEW GROUP-TRAINING PROCEDURE FOR HABITUATION DEMONSTRATES THAT PRESYNAPTIC GLUTAMATE RELEASE CONTRIBUTES TO LONG-TERM MEMORY IN C. ELEGANS. 2002. In Learning & Memory 102:369-379.

Experiments in sections 3.1 were published as: A NEW GROUP-TRAINING PROCEDURE FOR HABITUATION DEMONSTRATES THAT PRESYNAPTIC GLUTAMATE RELEASE CONTRIBUTES TO LONG-TERM MEMORY IN C. ELEGANS. 2002. In Learning & Memory 102:369-379.

Experiments in sections 3.2-3.4 were published as: GLR-1, A NON-NMDA GLUTAMATE RECEPTOR HOMOLOGUE IS CRITICAL FOR LONG-TERM MEMORY IN C. ELEGANS. 2003. In The Journal of Neuroscience, 23: 9595-9599.

*Karla Kaun is responsible for Experiment 3.2A and 3.3A and Sylvia Chen is responsible for Experiment 3.3b.

Experiments in sections 5.1-5.4 have been submitted for publication as: BLOCKADE OF MEMORY RECONSOLIDATION REVERSES A CELLULAR MECHANISM OF MEMORY. *In Nature, July 14, 2005.*
REFERENCES


Figure 24. Confocal Images 24 Hours After Distributed Habituation Training in worms that Received with Sham Shock or Heat Shock Immediately Following Each Training Block. Scale = 20 μm.
Figure 25. *avr-14* and *avr-15* mutant strains show 24-hour long-term memory after distributed training. Mean reversal magnitudes (±SEM) during testing. The *avr-14* gene codes for a glutamate-gated chloride channel and is expressed presynaptically on the mechanosensory neurons in the tap-withdrawal circuit. The *avr-15* gene codes for a similar channel but is expressed postsynaptically on the interneurons of the tap-withdrawal circuit (Dent et al. 1999)*=p<0.05. 
Figure 26. The *nmr-1* mutant strain shows 24-hour retention after distributed habituation training while the *nmr-1;glr-1* double mutant strain does not. Mean reversal magnitudes (±SEM) expressed as percent control response. **=*p<0.01.