THE EFFECTS OF ETHANOL ON SHORT-TERM AND LONG-TERM MEMORY

IN CAENORHABDITIS ELEGANS

by

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ABSTRACT

In this thesis I have used the model organism Caenorhabditis elegans to investigate the effects of ethanol exposure on learning and memory. In the first part of this thesis I identified how ethanol affects the formation of long-term memory for habituation training. I administered ethanol during long-term memory training and found that high doses of ethanol significantly impair the formation of long-term memory. Next, I examined if ethanol was having an effect on the kinetics of short-term habituation and I found that ethanol exposure significantly altered the rate of habituation when stimuli were administered at longer interstimulus intervals (ISI) but was relatively unaffected at shorter ISIs. Interestingly, we found that the effect of ethanol on long-term memory formation was dissociable from the impairments on the rate of habituation and was not a state-dependent nor context-dependent deficit. Further, increased tolerance to ethanol did not rescue this deficit in memory formation and ethanol exposure did not disrupt previously formed memories. Since glutamatergic neurotransmission has been shown to be disrupted by ethanol exposure and the role of glr-1, a non-NMDA-type glutamate receptor subunit, in long-term memory for habituation has been extensively researched, I investigated whether the effects of ethanol on long-term memory formation involves glr-1. Using a transgenic strain of worms that has GLR-1 tagged with a green fluorescent protein (GLR-1::GFP) I found that ethanol exposure results in an increase in the amount GLR-1::GFP along the posterior ventral nerve cord. Further, 24 hrs following habituation training, trained unexposed worms show decreased levels of GLR-1::GFP while ethanol exposed trained worms are not significantly different from control groups. This result suggests that ethanol exposure not only causes increases in the level of GLR-
1::GFP but also causes changes in glr-1 regulation that is normally associated with memory formation. In this thesis I have demonstrated that *C. elegans* is an ideal model system in which to study the effects of ethanol on learning and memory and have uncovered some important mechanisms mediating these effects.
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INTRODUCTION

Alcohol Impairs Memory Function

Alcohol is one of the most widely abused drugs in the world. In Canada, the total cost to the government of alcohol abuse is 14.6 billion dollars, which is over one-third of the total cost of all substance abuses (Taylor et al., 2007). Over three-quarters of our population consumes alcohol at least once per year and over 600,000 Canadians are alcohol-dependent (Rehm et al., 2006). Such high rates of alcohol consumption and abuse have a significantly negative impact on the health of our population. Alcohol abuse can lead to a number of negative health effects including increased risk of stroke and cancer, impaired mental functioning, and can lead to a number of physical and mental abnormalities in offspring if alcohol is consumed during pregnancy (Green, 2007; Klatsky, 2007). Some of the most significant effects of heavy alcohol consumption are cognitive and neuropsychological deficits.

Mental impairments from alcohol consumption can include deficits in decision-making, problem solving and learning and memory (Leckliter and Matarazzo, 1989; Selby and Azrin, 1998). The association between alcohol and memory in human subjects has been extensively studied and researched. For example, individuals who are dependent on alcohol show impaired performance on tasks such as learning word lists (Grant, 1987), short- and long-term logical memory (Selby and Azrin, 1998), and general working memory (Ambrose et al., 2001). A large body of research has focused on the impact of alcohol on memory however there is no single coherent theory as to the mechanism of action by which alcohol affects cognition. Figure 1 depicts the well-established model of memory formation, storage and retrieval that was originally
proposed in 1968 by Atkinson and Shiffrin; this model is often used to characterize the effects of alcohol on memory (White, 2003). In this model of memory, sensory information is transferred immediately to short-term memory (STM) and from there, depending on a number of factors including attention, pattern of stimuli presentation and motivation, information kept in short-term memory may be transferred or consolidated into long-term memory (LTM). The information kept in LTM storage can then be accessed and retrieved by placing it back in STM.

Figure 1. A general modal model of memory originally proposed by Atkinson and Shiffrin in 1968. Using this model of memory, it has been found that alcohol affects all processing stages to some degree. Alcohol intoxication results in impairments in the ability to retain information in short-term memory for longer than a few minutes. It also impairs the ability to transfer memory from short-term storage to long-term storage. However, it does not appear affect the retention of previously learned information (adapted from White, 2003).

Using this model of memory processing, the effects of alcohol on the transfer of memory between each stage has been investigated. The effects of alcohol on short-term memory have been somewhat contradictory, in part because of the variable operational definitions of short-term memory (Neath, 2004), the modality used to encode relevant stimuli (Duis et al., 1994) and the delay between the time stimuli are administered and then recalled (Stout and Murray, 2001). However, some important general effects on
short-term memory have been established. Acheson et al. (1998) found that ethanol significantly impaired their subjects’ performance on immediate and 20 min delayed recall of semantic word lists, though immediate recall was less severely affected compared to delayed recall. Other groups have found that intoxicated subjects can repeat new information immediately after presentation and can keep that new information in short-term memory storage for periods ranging from one minute up to a few minutes at which time recall becomes significantly impaired (Nordby et al., 1999).

Even though there are some inconsistencies in the literature on the ways that alcohol affects short-term memory, one of the most consistent findings in this field is that alcohol has a negative impact on the formation of new long-term memories. Alcohol attenuates the ability to transfer or consolidate short-term memories into long-term memory storage (White, 2003). This is the case for a number of different stimuli including word lists, number combinations, and new faces. Another hallmark feature of the effects of alcohol on memory is that the effects of alcohol on memory are dose-dependent. Ryback (1971) showed that when blood alcohol concentrations (BACs) are low there are small to moderate effects on the formation of long-term memory. However, when BACs are relatively high they found that memory impairments become much more severe, sometimes resulting in complete amnesia for the intoxication period (Ryback, 1971).

Human research has greatly increased our understanding of how alcohol interferes with memory function and storage but this research has not been able to elucidate the specific molecular mechanisms that underlie these memory deficits. The use of a variety
of model systems has led to the discovery of a number of different ways in which alcohol may impair memory processing.

**The Effects of Ethanol on Mechanisms that Underlie Memory**

Mammalian studies have shown that ethanol administration has different effects on learning depending on the type of learning task. Specifically, acute ethanol administration has been shown to significantly inhibit spatial learning similar to hippocampal lesions (Matthews et al., 1999; White et al., 2000), however non-spatial memory, like social recognition memory, is relatively unaffected or even facilitated by acute ethanol administration (Mikolajczak et al., 2001). To further test whether ethanol specifically disrupts hippocampal-dependent learning, Weitemier and Ryabinin (2003) tested the effect of moderate to high doses of ethanol on a type of learning that is not hippocampal-dependent (delay fear conditioning) and one in which it is (trace fear conditioning). They showed that moderate doses of ethanol selectively impaired learning of trace fear conditioning but not delay fear conditioning indicating that ethanol specifically impairs hippocampal dependent tasks.

Interestingly, both long-term potentiation (LTP) and long-term depression (LTD), which are thought to be cellular models for learning and memory, are strongly associated with spatial learning and other hippocampal-dependent tasks and are attenuated by ethanol exposure (Chandler, 2003; Izumi et al., 2005). Most forms of LTP and LTD are mediated by the activation of the N-methyl-D-aspartate (NMDA) receptors and, therefore, it is not surprising that ethanol has been found to block NMDA-evoked hippocampal neural activity (Crews et al., 1996; Diamond and Gordon, 1997). Further, chronic ethanol administration has been found to increase the levels of a number of NMDA
receptor subunits (Chandler et al., 1999). It appears that the increase in a specific NMDA receptor subunit, NR2B, is much more significant and long lasting in cortical neuron cultures compared to all other NMDA receptor subunits (Sheela Rani and Ticku, 2006). This has led to the popular hypothesis that the NMDA receptor and specifically the NR2B subunit is one of the primary targets of ethanol and the effects on this subunit may result in the ethanol-induced impairments on memory formation.

There is also ample evidence that the GABA\(_A\) receptor, the most abundant inhibitory neurotransmitter receptor in the mammalian nervous system, plays a major role in mediating the behavioral effects of ethanol. Using a number of \textit{in vitro} and \textit{in vivo} models, ethanol has been shown to potentiate GABA\(_A\) receptor activity and increase their sensitivity (Harris et al., 1998). This effect appears to primarily depend upon subunit composition, similar to the NMDA receptor (Boehm et al., 2004). It has been proposed that chronic ethanol treatment may result in enduring pre- and/or post-synaptic changes in GABAergic neurotransmission, which could counteract NMDA-mediated LTP induction. This is based upon evidence that shows that differences in LTP between ethanol treated and control groups are abolished by administration of picrotoxin (Schummers and Browning, 2001). Further, low concentrations of ethanol potentiate GABA-mediated inhibition in hippocampal pyramidal cells, following stimulation (Weiner et al., 1997). Like NMDA receptors, ethanol alters the expression levels of specific GABA\(_A\) receptor subunits but in this case some subunit expression is increased while others are decreased (Sheela Rani and Ticku, 2006). Taken together, these data suggest that ethanol has a fast-acting component that can result in modulating LTP/LTD induction by altering ion channel function but also ethanol can alter the physiological state of the system through
slower-acting changes in the receptor density and subunit substitution of both excitatory and inhibitory receptors.

In addition to the identification of the mechanisms by which ethanol affects receptor function and expression, using genetically modified mice and genetic mapping techniques researchers have been able to identify a number of genes that increase or decrease susceptibility to behaviors associated with ethanol consumption. Interestingly, a large number of papers have converged on evidence showing that ethanol consumption and associated behaviors involve a number of different genes linked to synaptic transmission. Using quantitative trait loci analysis, Fehr, et al. (2005) found that ethanol-drinking preference, a behavior in mice that is thought to mimic alcohol addiction in humans, is linked to the gene *Stxbp1*, which encodes a Sec1/Munc18-type protein essential for neurotransmitter release. Using D1 and D2 receptor knockout (KO) mice, the importance of the neurotransmitter dopamine in mediating some of the behaviors associated with ethanol consumption has also been shown. D1 KO mice have been shown to exhibit decreased preference for ethanol and D2 KO mice also exhibit decreased preference for ethanol but also decreased self-administration of ethanol and an absence of condition place preference to ethanol (El-Ghundi et al. 1998; Thanos et al. 2005; Risinger et al. 2000; Cunningham et al. 2000). These data show that using different genetic techniques it is possible to identify some of the genes that are important in neurotransmission that also play a role in mediating some of the behaviors associated with ethanol consumption.

The use of simpler invertebrate model systems has allowed researchers to identify a number of shared sites of the action of ethanol between vertebrates and invertebrates,
and has led to the discovery of a number of important proteins and mechanisms that are affected by ethanol. Most importantly, researchers have been able to directly link these proteins and mechanisms to distinct behavioral effects. For example, using the model system *Drosophila melanogaster*, one of the most studied organisms in biology, researchers have been able to show that *D. melanogaster* exhibit significant behavioral deficits including impairments in locomotion and postural control when exposed to ethanol (Parr et al., 2001). Using these behaviors Moore et al. (1998) was able to perform a genetic screen for genes that may regulate sensitivity to ethanol and identify novel molecular targets of ethanol. They identified a mutant that had increased sensitivity to ethanol and, interestingly, the genetic mutation responsible for this effect was found to disrupt the *amnesiac* gene, a gene originally identified in screen for learning and memory deficits (Moore et al., 1998). This gene is critically important for adenylate cyclase and cAMP-dependent protein kinase (PKA) signaling. Alterations in ethanol sensitivity could be rescued by pharmacological activation of adenylate cyclase or PKA in adult flies, further supporting the fact that this signaling pathway plays an important role in mediating the acute effects of ethanol on behavior. Interestingly, similar overlaps in genes that play a role in learning and memory and those that regulate sensitivity to ethanol first identified in invertebrates also occurs vertebrates (Wolf and Heberlein, 2003). In 2003 Davies et al. identified the gene *slo-1* in *Caenorhabditis elegans*, which encodes a BK potassium channel as a primary target of ethanol in the nervous system. Since then a number of different groups have shown that in mammalian systems, similar BK channels are modulated by ethanol and that the subunit composition of this channel
confers ethanol sensitivity of different brain areas (Martin et al., 2004; Pietrzykowski et al., 2004)

**Investigations into the Effects of Ethanol in *Caenorhabditis elegans***

The use of the model system *C. elegans* has led researchers to important discoveries into identifying direct targets of ethanol action. *C. elegans* is an ideal species to use as a model system to study a variety of biological and behavioral mechanisms. Bacterial geneticist Sydney Brenner first introduced *C. elegans*, a tiny, transparent nematode as a model organism for genetic studies in the 1960s. Since, then this species has come to be one of the most studied species in the world for a number of different reasons. The entire animal consists of only 959 cells, of which 302 are neurons with approximately 5000 chemical synapses, 600 gap junctions, and 2000 neurochemical junctions for which a complete wiring diagram is available (Chalfie et al., 1985). Further, the *C. elegans* genome has been completely mapped and sequenced. Research using this system has been integral in elucidating a number of important biological mechanisms including the characterization of programmed cell death and the discovery of RNA interference; both of these discoveries have led to the awarding of Nobel Prizes. Since its introduction as a genetic model system, *C. elegans* has been used for a number of other research applications including development, basic genetics and, more recently, behavior.

![Diagram of *Caenorhabditis elegans*](image)

**Figure 2. Caenorhabditis elegans.** This is a representative image of a 4 day-old hermaphrodite worm. (image adapted from www.wormbase.org)
For all of the reasons listed above *C. elegans* was chosen by Davies et al. (2004) as an ideal model system in which to study the effects of ethanol. Tissue concentrations of ethanol that are found in intoxicated humans have been reproduced in *C. elegans* and have been shown to produce neurodepressive effects on a number of behaviors in this species (Davies et al., 2003). Davies et al. (2003) showed that mutations in *slo-1*, a gene that encodes the pore-forming α-subunit of the BK potassium channel, conveyed significant resistance to the effects of ethanol on two observable behaviors, locomotion and egg-layings. Further, rescue experiments and direct SLO-1 physiological current measurement experiments were performed that showed that SLO-1 was a direct target of ethanol *in vivo*.

When *C. elegans* are exposed to ethanol, an acute adaptive response is observed which mimics a similar adaptive response observed in vertebrate systems, including humans (Davies et al., 2004). A screen for genes that are responsible for tolerance to ethanol revealed that *npr-1*, a gene that has a relatively high homology to the human neuropeptide Y (NPY) gene, negatively regulates acute tolerance to ethanol (Davies et al., 2004). Davies et al. (2004) found that worms with mutations in *npr-1* are able to recover locomotor speed while exposed to ethanol much more quickly than wild-type worms. Natural variations in this gene also confer increased tolerance to the neurodepressive effects of ethanol on locomotion. The NPR-1/NPY biochemical pathway is highly conserved and plays an important role in responses to ethanol in a number of species, which suggests that natural variations in this gene may be responsible to the
variability of ethanol tolerance in natural populations, including *C. elegans* populations (Thiele et al., 2000; Wu et al., 2003).

To date there is only a single published study of the effects of ethanol on behavioral plasticity in *C. elegans*. Bettinger and McIntire (2004) found a link between the memory for chemosensory adaptation and ethanol exposure. They found that adaptation to a chemotaxic stimulus was a state-dependent process, in that, if previous adaptation to the stimulus occurred during ethanol administration, then later recognition of that stimulus would only occur if tested in the presence of ethanol. This result shows that ethanol has an effect on memory processing in *C. elegans*. Further, they found that worms with mutations in genes that are important for dopamine transmission were impaired in state-dependent learning, identifying a novel mechanism in which dopamine neurotransmission effects state dependent memory in *C. elegans*.

**Caenorhabditis as a Model System to Study Learning and Memory**

Not only is *C. elegans* a very tractable genetic model system it is an ideal species in which to study the cellular mechanisms involved in learning and memory. *C. elegans* have been shown to exhibit a number of different forms of learning and memory including habituation, short- and long-term memory (Rankin et al., 1990), context conditioning (Rankin, 2000), and differential classical conditioning (Wen et al., 1997). One of the most studied behaviors of *C. elegans* is that of habituation, which has been defined as a decrement in response to repeated stimuli that cannot be attributed to adaptation or fatigue. Habituation is a one of the simplest forms of non-associative learning and has been shown to occur in every organism studied. In a pioneering study Rankin and Broster (1992) showed that *C. elegans* shows a decrement in reversal
magnitude in response to non-localized, mechanical stimuli and that this form of habituation exhibited all of the characteristics that were set out earlier by (Groves and Thompson, 1970). Interestingly, Rankin and Broster (1992) found that the interstimulus interval (ISI) had a number of effects on the kinetics of habituation including altering the rate of habituation, the asymptotic level, and the rate of recovery following habituation (Fig 2). These results led Rankin and Broster (1992) to hypothesize that the cellular mechanisms that underlie habituation at both the 10s ISI and the 60s ISI include some of the same mechanisms but also include mechanisms that are distinct to each ISI.

![Graph](image)

**Figure 3. The Effect of ISI on Rates and Patterns of Habituation.** Reversal responses of wild-type worms shown as the mean percent initial response across 30 tap stimuli with three recovery taps given at 30 s, 5, and 10 min after habituation training. The 10-s ISI group shows more rapid habituation and a lower asymptotic level when compared to the 60-s ISI group. The 10-s ISI group also shows faster and more rapid recovery following habituation training than the 60-s ISI group.

Following the initial investigations into the dynamics of STH a number of critical components of STH have been identified. Using laser ablation techniques and previously mapped neural connections; Wicks and Rankin (1995) were able to uncover the neural circuit that underlies the tap-withdrawal response, which involves five
that encodes a glutamate transporter and is expressed in the mechanosensory neurons, had significantly altered STH. This result indicates that glutamatergic neurotransmission is a critical component of STH, which parallels the importance of glutamatergic neurotransmission in most models of learning and memory.

Figure 4. Simplified Neural Circuit Underlying Touch Withdrawal. The circuit consists of five mechanosensory neurons (triangles), eight command interneurons (circles), and two motor neuron pools (squares). All cells represent bilateral classes of cells except ALM, which is a single cell. The arrows and dotted lines represent chemical synapses and gap junctions, respectively. The number of synaptic contacts is proportional to the width of the arrows. The gray arrows indicate the synaptic connections that have been hypothesized to be the sites of plasticity that mediate short-term habituation (adapted from Butterfield and Rankin, in press).

This tap paradigm has been proposed as a way to assess a form of short-term memory because the response decrement depends upon the ISI, and like in other species and models of memory, the more often and rapid stimuli are administered, the faster learning occurs (Siddle et al., 1985). For a number of reasons, some of which have been detailed above, short-term habituation (STH) in C. elegans is an ideal model to study both non-associative learning and short-term memory.

C. elegans can also retain memory of habituation training for at least 24 hours and is considered to be a model of long-term memory (LTM). Memory for habituation
C. elegans can also retain memory of habituation training for at least 24 hours and is considered to be a model of long-term memory (LTM). Memory for habituation training or long-term habituation (LTH) has been extensively researched at the behavioral, biological and genetic levels. LTH is influenced by the spacing or distribution of training and is protein synthesis dependent. Rose et al. (2003) found that one of the AMPA/KA-type glutamate receptor subunits present in C. elegans, GLR-1, is critical to the induction and maintenance of LTH. They found that mutations in glr-1 or blockade or AMPA/KA-type receptors using DNQX resulted in the inability to form LTM for habituation training. Further, using a reporter gene that has a green fluorescent protein tagged to GLR-1 (GLR-1::GFP) they were able to show that the presence of LTH is accompanied by a decrease in the amount of GLR-1::GFP. More recently, Rose and Rankin (2006) found that this form of memory is sensitive to disruption by reconsolidation blockade.

Many cellular models of memory in mammals require the involvement and activation of NMDA receptors that are composed of NR1 subunits that are required for active, surface-expressed receptors and a combination of one or more NR2A-D subunits. However, the expression of long-term memory for habituation training is not impaired in worms that have a mutation in nmr-1, the C. elegans homologue of the mammalian NMDA receptor subunit NR1 (Rankin, et al., unpublished results). It has been shown that the induction of LTD can be NMDA receptor-independent depending on the induction protocol, brain region and type of neuron studied (Malenka and Bear, 2004). The significant amount of information known about LTH and the striking similarities between LTH and the mechanisms that underlie memory processing such as the
trafficking of AMPA receptors in LTD/LTP makes *C. elegans* an ideal model system in which to study long-term memory.

The multitude of factors that influence both STH and LTH in *C. elegans* provide a model system in which we may examine and identify key components that are important in learning and memory and, more importantly for this thesis, this system allows for the identification of ways that ethanol affects learning and memory at the cellular level.

**Thesis Objectives**

In this thesis, I carried out a research program to address two major objectives in order to further our understanding of how ethanol affects not only memory processing but also some of the molecular mechanisms that underlie these effects. The first objective was to determine what, if any, effect ethanol has on short- and long-term memory processing. To assess the effects of ethanol on long-term memory the basic protocol involved exposing worms to ethanol during the encoding or training phase and then testing the worms in the absence of ethanol. I hypothesized that ethanol exposure would impair memory formation in *C. elegans* as it has been shown to impair memory in other species that have been studied. Utilizing a number of different modified protocols and a mutant strain of worms, I hypothesized that I would show that ethanol has a specific effect on long-term memory formation. To test the effects of ethanol on short-term memory I used a modified version of the short-term habituation paradigm that had been previously established by Broster and Rankin (1990) to study short-term memory. Because previous research showed that when stimuli were given at different frequencies changed the rate of habituation and may recruit different cellular mechanisms I included experiments where worms were tested in the presence of ethanol and administered tap
stimuli at two different frequencies in order to gain further insight into how ethanol
effects short-term memory. Finally, I hypothesized that by using a number of strains of
worm with mutations in genes already implicated in mediating the effects of ethanol on
*C. elegans* behavior I would be able to identify some of molecular mechanisms that
underlie any behavioral deficits I would observe.

A number of different studies have converged on the importance of glutamate
receptors in mediating the effects of ethanol and also in underlying memory formation.
Thus, my second objective was to investigate how alcohol exposure effects levels of *glr-1*
by using a strain of worms carrying a GLR-1::GFP tagged reporter gene to gain further
understanding of how glutamate receptors are affected by ethanol and how this may
result in memory deficits. By accomplishing these objectives, this research has led to the
identification of genes and/or mechanisms that mediate the some of the behavioral effects
of ethanol and it has important implications for determining susceptibility to alcohol-
induced and alcohol-related disorders.
MATERIALS AND METHODS

Subjects

Worms were maintained on nematode growth medium (NGM) agar seeded with *Escherichia coli* (OP50; Brenner, 1974). *C. elegans* wild-type Bristol (N2) and *npr-1*(ad609) strains were obtained from the *Caenorhabditis elegans* Genetic Center (University of Minnesota, Minneapolis, MN). To investigate the role of specific genes on modulating the effects of ethanol on behavior we obtained the *avr-14*(ad1302) strain from J. Dent (McGill University, QC). To quantify changes in glutamate receptor (GLR) subunit expression, we used a transgenic *C. elegans* strain carrying a green fluorescent protein fusion to GLR-1 (GLR-1::GFP) that was obtained from J. Kaplan (Harvard University, Boston, MA).

Apparatus

Behavioral observations of individual worms were made with a Wild Zeiss stereomicroscope (Wild Zeiss Canada) and attached video-recording equipment (Panasonic D5000 camera, Panasonic AG-1960 video cassette recorder, and JVC color monitor). The timing of the stimulus was done using a superimposed stopwatch onto the video screen, which was created by a Panasonic 814 time-date generator.

In the experiments outlined in Chapter I the Petri plates containing 15-20 worms were placed in a holder in such a way that a tap (1.5 N force) could be delivered to the side of the Petri plate by a copper rod connected to the armature of a 6-V electromagnetic relay. This tapper was activated with a Grass S88 (Quincy, MA) stimulator set to deliver a single tap when needed. A Marzhauser micromanipulator (MM33) was used to
smoothly move the plate holder on the stage of the microscope while still keeping it in view of the camera field (Fig 4).

**Figure 5. Schematic of the Behavioral Testing Apparatus.** Agar-filled Petri dishes containing the test subjects are placed in a holder on the microscope stage. A micromanipulator attached to the holder allows for movement of the dish around the stage. Also attached to the micromanipulator is an electromagnetic relay connected to a copper rod that delivers the non-localized tap to the side of the Petri plate when an electric pulse is sent from the Grass S88 stimulator. The behavior of the worms is recorded from a camera attached to the microscope and is relayed to a video monitor.

**Procedure**

**Plate preparation**

In each of the experiments, NGM agar Petri plates were prepared 2 days prior to the training procedure. One day before the day of testing the Petri plates were seeded with a small amount of *E. coli* and were left to dry so that the plates would only contain a small circle of *E. coli* that was adequate to feed a group of 15-20 worms for ~48 hrs. On
the day of training, single plates were weighed and the mass of the agar in the plate was determined. The ethanol (100%) was added to the agar to the desired concentration (0.2M, 0.4M, 0.6M), the plates were sealed with parafilm, and left for 1-2 hours to allow equilibration of ethanol into the agar. At this point experimental worms were transferred to these plates.

**Long-term Habituation**

At approximately four days of age worms were transferred to either ethanol plates containing the specified concentration of ethanol or ethanol-free plates, allowed one hour to adapt to the new environment and then training commenced. To deliver the same amount of mechanosensory stimuli to a large number of groups, plates containing the training groups were placed in a plastic container and dropped from a height of 5cm onto a tabletop. Training of the worms consisted of the delivery of 4 periods of mechanosensory stimuli (20 drops given at a 60s ISI) separated by 1 hour break periods. Control groups were placed in the same plastic container and were dropped at the same time as the last stimulus was given to the trained groups. One hour following the last stimulus, all subjects were transferred to an ethanol-free plate containing a small amount of *E. coli*.

The groups were tested 24 hours later for the retention of the habituation training. Plates containing trained or control groups were placed in the tapping apparatus and each group was subjected to 5 taps (Fig 5). The responses to each tap stimuli were collected using a random sampling technique.
Figure 6. Basic Protocol for Investigating the Effects of Ethanol Exposure on the Induction of Long-term Habituation. One hour before habituation training, approximately 40 worms are transferred to two different ethanol treated Petri plates, one group of 20 worms will be the control group and one group of 20 worms will be the trained group. Worms are given a one hour rest period before the commencement of training. Training consists of 4 sets of 20 box drops given at a 60s ISI (unless otherwise stated), each separated by one hour rest period. Following the training procedure both groups are transferred to ethanol free plates and the responses to 5 test taps 24 hrs later are recorded.

Short-term Habituation Testing

Fifteen four-day-old worms were transferred using a platinum pick to either a NGM agar plate containing ethanol or a NGM agar plate without ethanol. The worms were given one hour to become accustomed to the new environment and to develop an internal tissue concentration of ethanol that was dependent upon environmental ethanol concentrations which has been previously been shown to occur in identical experimental conditions (Davies, et al., 2002). The plates were then placed in the plateholder on the microscope stage and the behavior of the worms was recorded using the video-recording equipment. After 6 min spent on the stage, worms were given 30 tap stimuli at either a 10s or 60s interstimulus interval (ISI).
Group Testing Protocol

In the behavioral experiments each response to tap that was recorded (the 5 test
taps in the LTH protocol and the 30 taps in the STH protocol) was collected using a
random sampling of responses from 5-10 worms from a plate containing 15-20 worms.
Prior to each tap the Petri plate containing the worms was moved around the microscope
stage in order to maximize the number of worms in the field of view and consequently
the number of responses recorded at the time of the tap. Using this method allowed me to
record the responses from a large number of worms but did not allow for the
measurement of repeated responses from the same animal. As a result, instead of using a
set number of animals as a consistent measure in each experimental group, I used a
threshold of approximately 15 responses to each tap as a consistent measure throughout
my experiments.

Scoring Data

After recording all the responses to the tap, the reversal response magnitude (i.e.
the distance the worm swam backwards in response to the mechanical tap stimulus
delivered to the side of the Petri plate) was scored using a stop-frame video analysis. The
documents containing these tracings of worm length and tap response magnitude were
scanned (UMAX Astra 2100U) into an iMac computer using Epson Perfection software.
The final measurements were determined by NIH Image software and then analyzed
using the statistical package, StatView 4.5. The graphical analysis of the rate of short-
term habituation was simplified by grouping responses to each stimulus into sets of 3.

Imaging Methods

At the designated time of imaging, worms were placed on a sterile glass
microscope slide in 12μL of 50mM sodium azide for paralysis. Worms were then placed on an agar pad and covered with a 1.5 thickness coverslip. Images were obtained using Olympus FV1000 confocal microscope equipped with five lasers (405, 442, 488/514, 568 and 633 nm). GFP was excited using a 488nm wavelength laser setting with the emitted light collected by passing through a ~510-550nm bandpass filter. Optical sections were collected at 0.5 μm intervals using a 60x oil lens.

The post-synaptic glutamate receptor marker GLR-1::GFP is present in both the nerve ring located in the head of the animal and along the ventral nerve cord, which runs from the head to the tail. GLR-1::GFP was most easily quantified along the ventral nerve cord; therefore, images were collected immediately posterior to the vulva along the ventral nerve cord. The total stacks of images of 15-25 optical sections for each ventral nerve cord segment were then summed into a single projection image in Image J v1.33. Images for GLR-1::GFP were consistently acquired using the same microscope settings: gain=~1.0; PMT=600 (+/-50), laser=~1.0%. The GFP expression in the ventral nerve cord is uniform in thickness; therefore, the total area of GFP expression and the number of clusters of GLR-1::GFP were measured using ImageJ v1.33.

Statistical Analysis

To investigate differences in the expression of LTH and differences in the total area of GLR-1::GFP observed I used two-way mixed ANOVAs. If the ANOVA resulted in a p-value of less than 0.05, a Fisher’s PLSD planned comparison was performed. In behavioral analyses of LTH, if the within group effect (the difference between control and trained) was significant (p<0.05) then LTM for habituation training was present. In
the imaging experiments a significant within group effect (p<0.05) showed that habituation training had an effect on GLR-1::GFP expression.

The statistical methods that were used to analyze short-term habituation have already been established through previous research investigating changes in habituation in *C. elegans* (Rankin and Broster, 1992). In general, to investigate the presence of habituation and differences between groups I performed two-way mixed ANOVAs with the between group variable being either dose of ethanol or strain and the within group variable being habituation across stimuli. If more than two groups were included in a dataset and significant between group effect was observed, I performed Fisher’s protected least significant difference (PLSD) planned comparisons to identify which groups were significantly different from each other. Statistical significance in all datasets was established when the p-value was less than 0.05.
RESULTS

Chapter I: The Effect of Ethanol Exposure on Long-term Habituation

1.1 Ethanol Impairs Long-term Habituation in a Dose-Dependent Manner

The first question I asked was whether doses of ethanol that are physiologically relevant to intoxication (0.4M, which is equivalent to 0.10 blood alcohol concentration in humans; Davies et al., 2003) interfered with the formation of LTM for habituation training. To do this I tested four groups; two non-ethanol groups, one control and one trained group, and two ethanol treated groups, one control and one trained group. The ethanol-treated groups were exposed to ethanol during the training phase of the distributed long-term habituation training protocol. Following the training period, worms were transferred to ethanol-free plates and the test responses of trained ethanol worms were compared to those of ethanol treated control worms that did not receive training on the previous day.

![Graph]

Figure 7. Ethanol Treatment Disrupts the Formation of LTM for Habituation Training. Exposure to 0.4M ethanol during the training phase of the LTH protocol results in trained and control groups exhibiting similar reversal magnitudes in response to tap 24 hrs after training. However, the non-ethanol trained group responds significantly less than the non-ethanol, control group.
An ANOVA for all four groups revealed a significant effect of the combination of ethanol treatment and habituation training ($F(3, 250)=2.893, p<0.05$) and a planned comparison showed that the average responses of the trained, non-ethanol group responded significantly less that the non-ethanol, control worms ($p<0.01$) indicating the presence of LTM (Fig 7). However, when we compared the average responses to tap 24 hrs after training for both ethanol trained and ethanol control groups we did not observe any significant difference ($p>0.05$), showing that the trained groups did not form LTM.

To investigate whether there was a dose-dependent effect on the formation of LTM we separated worms into 8 different groups, two non-ethanol groups (0.0M) and 6 groups given different doses of ethanol (0.2M, 0.4M or 0.6M) during control and habituation training. An overall ANOVA showed significance between the combination of dose and training ($F(7, 332)=2.603, p<0.05$; Figure 8). A Fischer’s post hoc test revealed that in the 0.2M dose groups trained worms responded significantly less that control worms ($p<0.05$) which showed that 0.2M ethanol exposure during training did not impair memory formation. However, no significant difference was observed between the trained and control animals that had been exposed to 0.4M ethanol ($p>0.05$). Similarly, at the 0.6M dose, no significant difference was observed between response magnitudes in trained and control groups ($p>0.05$). This result showed that there was a dose-dependence on the formation of memory, with relatively higher doses inducing memory impairments.
Figure 8. The Dose-Dependent Effect of Ethanol on the Formation of Long-term Memory for Habituation Training. In both the naïve condition (0.0M) and low ethanol dose condition (0.2M) trained worms respond significantly less than trained worms showing the presence of memory. At the higher doses (0.4M, 0.6M), we do not observe any differences in the response magnitude between control and trained groups, showing that the ethanol-treated trained groups were unable to form LTM.

1.2 Ethanol Selectively Alters Habituation at Longer Interstimulus Intervals

One possibility is that ethanol exposure impaired the formation of memory by affecting the acquisition or encoding of habituation during the training blocks in the long-term habituation protocol. To assess this possibility I examined the effect of ethanol on the kinetics of habituation at a 60s ISI (the ISI at which habituation training is administered). Figure 9 shows that at a 60s ISI worms habituated in the presence of ethanol showed a significantly slower rate of habituation and responded at a much higher asymptotic rate when habituated, a pattern of STH that has never before been observed. To test whether dosage had an effect on STH at a 60s ISI we administered the STH protocol in the presence of both 0.2M and 0.4M ethanol. An overall ANOVA on habituation given at a 60s ISI revealed a significant effect of stimulation ($F_{(9,378)}=23.839$, $p<0.001$) showing that all groups habituated to the tap stimuli. The
results from the ANOVA also revealed that there was a significant main effect of ethanol dose \((F(2,42)=4.169, p<0.05)\) and a significant interaction between ethanol dose and the rate of habituation \((F(18,378)=1.947, p<0.05)\). A Fischer’s post hoc test revealed a significant difference between the naïve group and the 0.2M group \((p<0.05)\) and between the naïve group and the 0.4M group \((p<0.01)\). Analysis of the initial and habituated responses of the groups reveals that there is no significant effect of ethanol dose between the groups \((F(2,49)=1.698, p>0.05)\) and overall there was no significant effect of dose between groups on responses at habituated level \((F(2,49)=2.114, p>0.05)\) but a Fischer’s post hoc test revealed that there was a significant difference between the naïve and 0.4M groups \((p<0.05; \text{Fig 9})\). These results show that ethanol at both the 0.2M and 0.4M dose impairs habituation however analysis of the effect of stimulation showed that all groups did show significant habituation. Interestingly, examination of the habituated level of all groups revealed that higher doses of ethanol have more significant effect on the asymptotic level of habituation.

**Figure 9. Comparison of Short-term Habituation Between Naïve an Ethanol-treated groups at a 60s ISI.** Comparison of the initial responses of all groups reveals that there is no significant difference between the three groups. However, naïve groups habituate significantly more than both ethanol-treated groups shown by a lower asymptotic level of response magnitude. There are not major differences between the behavior of the ethanol treated groups but both ethanol-treated groups differ significantly from the naïve group. Examination of the response level in the last set of responses reveals a significant difference between the naïve group and the 0.4M ethanol treated group.
When tap stimuli are administered at different ISIs differences are observed in both the rate of habituation and asymptotic level of habituation, a finding that led Rankin and Broster (1992) to suggest the hypothesis that there are multiple mechanisms that underlie habituation, some of which are recruited by longer ISIs and some of which are recruited by shorter ISIs. To test whether ethanol has a specific ISI-dependent effect on habituation, I administered the short-term habituation protocol at a 10s ISI in the presence and absence of 0.4M ethanol. I found that both ethanol and control groups habituated at very similar rates and reached a very similar asymptotic level of responding once habituated. An ANOVA showed a significant effect of stimulation \((F (9,153)=9.363, p<0.001)\) indicating that both groups habituated. However, there was no significant effect difference between the ethanol treated and naïve groups \((F (1,17)=0.026, p>0.05)\).

Figure 10. Comparison of Short-term Habituation Between Naïve an Ethanol-treated groups at a 10s ISI. The initial responses to tap stimuli are not significantly different as seen by the comparison of block 1 between each group. The rate of habituation and the asymptotic level following habituation are not statistically different.
1.3 Ethanol has a Specific Effect on the Formation of Long-term Memory

Up to this point I have shown that ethanol negatively affects both short-term and long-term memory, however I have not shown whether these are two independent processes or whether the impairments in short-term memory lead to the lack of long-term memory. To address this issue I utilized the unique attributes of the mutant strain, *avr-14 (da1371)*, in an altered LTH paradigm.

The *avr-14* gene encodes an α-type subunit of a glutamate-gated chloride channel. These types of channels are only found in invertebrates, including insects and nematodes but common structural properties have classified these channels into the same channel family as GABA, glycine, and nicotinic acetylcholine receptors (Cully et al., 1994). In the tap-withdrawal circuit *avr-14* is expressed exclusively in the mechanosensory neurons and this gene has been shown to play an important role in mediating LTH to short ISIs. In wild-type worms, the formation of LTM for habituation is highly dependent upon the ISI at which training occurred; LTM is observed when
training is given at a 60s ISI but not at a 10s ISI (Steidl et al., in preparation). However, our lab has found that worms carrying a mutation in *avr-14* can form LTM when habituation training is given at a 10s ISI (Steidl et al., in preparation).

![Graph showing the effect of ethanol treatment on memory formation.](image)

**Figure 11. Ethanol Treatment Affects the Formation of Memory.** *avr-14* mutants that were given habituation training at a 10s ISI respond significantly less in response to tap than control groups. When ethanol was administered during 10s ISI habituation training, no significant difference was observed between trained and control groups.

Since ethanol exposure impairs STH at a 60s ISI but not at a 10s ISI I hypothesized that I could dissociate the effects of ethanol on STH and LTH by exposing *avr-14* worms to ethanol during LTH training at a 10s ISI. To test this hypothesis, I administered the LTH training protocol previously described to both naïve and ethanol exposed *avr-14* worms and wild-type worms with the exception that the stimuli were given at a 10s ISI rather than at a 60s ISI. If ethanol blocks the formation of LTM at a 60s ISI because of deficits in STH at a 60s ISI, then ethanol exposed *avr-14* worms should show LTH after training at a 10s ISI. However, if ethanol has dissociable effects...
on STH and LTH then *avr-14* worms should not show LTM when exposed to ethanol during 10s ISI training. An overall ANOVA revealed that there was no main effect of training and strain (F (7,441)=1.554, p>0.05). However since I was specifically interested in a planned comparisons between the *avr-14* trained and control groups, a Fischer’s PLSD was performed. Figure 11 shows and a Fischer’s post hoc test showed that *avr-14* mutants do not form LTM when trained in the presence of ethanol at a 10s ISI (p>0.05) but do form LTM in the untreated condition (p<0.05). This result indicates that although ethanol does effect STH at a 60s ISI, this effect is dissociable from the effects that ethanol has on the formation of long-term memory.

1.4 Memory for Habituation Training is Not State-Dependent or Context Dependent

Another possibility is that we do not observe the presence of LTH when worms are exposed to relatively high concentrations of ethanol during habituation training because of either state-dependent or context-dependent effects (Rankin, 2000). Rankin (2000) showed that when worms were trained and tested in the presence of a contextual cue, they showed greater retention of the training than worms trained and tested in different contextual environments. Bettinger and McIntire (2004) showed that when worms were adapted to an olfactory stimulus in the presence of ethanol, memory for the olfactory stimulus or adaptation to that stimulus only occurred in the presence of ethanol, showing that *C. elegans* are capable of state-dependent learning. Because of this, I wanted to investigate the possibility that the lack of LTM observed in our experiments was because the worms were not in the same context or not in the same state during testing as during training. To test this I trained animals in the presence of an intoxicating dose of ethanol (0.4M), removed them after the training and then re-exposed them during
the testing period to either a similar dose of ethanol (0.4M) or a dose of ethanol that would remind the animals of the context in which they were trained (0.05M; strong enough to have chemosensory cues of ethanol, but not strong enough to alter state). An overall ANOVA showed that there was a significant main effect of ethanol exposure and training (F(5,378)=2.813, p<0.05; Fig 12). Planned comparisons revealed that responses of worms that were trained and tested in all other conditions (0.4M, 0.05M) were not significantly different from untrained worms (p>0.05). Thus, we did not find any evidence for the presence of state-dependent or context-dependent memory for habituation training with a dosage of 0.4M or 0.05M ethanol, respectively. This result confirmed that the lack of LTM that we observed was not due to an effect of context or state dependence.

Figure 12. The Formation of Memory for Habituation Training is Not State-dependent or Context-dependent. To test whether worms were able to show the presence of memory in the same state that they were trained in worms were trained in a 0.4M ethanol environment and similarly tested in the same 0.4M environment. No significant difference was observed between trained and control groups in this condition, showing that LTM for habituation training is not state-dependent.
Increased Tolerance to Ethanol Does Not Rescue Memory Deficits

Davies et al. (2004) showed that in *C. elegans* natural variation in the *npr-1* gene regulates acute tolerance to ethanol. They also found that animals lacking *npr-1*, which encodes for NPR-1, a neuropeptide Y-like receptor, recovered from the locomotory effects of ethanol more quickly than wild-type worms. If NPR-1 negatively regulates acute tolerance, a neuroadaptive process that compensates for the effect of ethanol, I hypothesized that *npr-1* mutants might be able to adapt more quickly to ethanol exposure and, if so would not experience the deficits in memory formation seen in wild-type worms exposed to ethanol. To test this hypothesis we tested the formation of LTM in both non-ethanol and ethanol (0.4M) groups (Fig 13).

![Graph showing comparison of reversal magnitude between non-ethanol and ethanol conditions](image)

**Figure 13.** Exposure to Ethanol During Training Blocks the Formation of LTM in *npr-1* Worms. To test whether increased tolerance to ethanol affected the impairment of LTM for habituation training, *npr-1* worms the responses of non-ethanol and ethanol treated worms were observed 24 hours following training. In the non-ethanol condition, there was significant difference between trained and control worms, showing that *npr-1* worms can form LTM. However, ethanol treated groups are not significantly different indicating that ethanol blocks LTM formation in *npr-1* worms.
An overall ANOVA showed a significant effect of dosage and training in the npr-1 mutants (F (3,284)=4.436, p<0.01). A planned comparison found that in ethanol-free conditions, trained npr-1 worms responded significantly less than control npr-1 worms (p<0.05), indicating that they were able to form LTM. But in ethanol conditions, the responses from control and trained npr-1 worms did not significantly differ (p>0.05) showing that the formation of LTM in npr-1 worms was impaired by ethanol exposure. This observation shows that even though npr-1 mutants can quickly adapt to ethanol exposure it does not protect them from the effects of ethanol on LTM.

1.6 Ethanol Does Not Disrupt Previously Formed Memories

To determine whether ethanol exposure alone or whether ethanol exposure specifically during training was responsible for the lack of LTM we separated worms into 2 different ethanol exposure groups; during training and after training. All groups were exposed for the same duration and the presence of memory was tested 24 hrs after the last block of the LTH training. Worms that were exposed to ethanol immediately after training showed a significant difference between trained and control groups, indicating the presence of LTM (F (3,204)=3.201, p<0.05, Fig 14).

**Figure 14. Ethanol Exposure After Habituation Training Does not Disrupt the Expression of Memory 24 hrs Later.** All groups were given habituation training in the absence of ethanol. Then the naïve groups were transferred to ethanol-free plates, and the experimental groups were transferred to 0.4M ethanol plates. Six hours later, all groups are transferred to ethanol-free plates and the presence of memory is tested 18 hours later. Both trained groups responded significantly less than the control groups showing that ethanol exposure after habituation training did not impair the expression of memory 24 after training.
A Fischer's post hoc test showed that in the naïve condition, there was a significant difference between the trained and control groups (p<0.05) and there was also a significant difference between the ethanol trained and control groups (p<0.05). This result confirmed that ethanol exposure during training impairs the ability to form LTM and also that ethanol exposure after training does not disrupt previously formed memories.

Discussion Chapter I

In Chapter I, I showed that ethanol exposure during habituation training results in impairment in the ability to form long-term memory for that training. In line with memory impairments in a number of other species, including humans, we found that the impairment in memory was dose-dependent. We found that at low doses, worms were still able to form memory for the habituation training. However, at high doses of ethanol (0.4M, 0.6M) we found that worms did not express memory 24 hours after habituation training. This finding was important in a number of ways. First, it is important because
it shows that in *C. elegans* ethanol impairs more complex behaviors than just locomotory speed and egg-laying. Also, it shows that the effects of ethanol on memory are dose-dependent, which is one of the critical features that is observed in most models used to study the effects of ethanol on memory, which helps to establish *C. elegans* as a good model system in which to study ethanol-induced impairments in long-term memory formation. The dose-dependent impairment could suggest a number of different mechanisms that underlie this result. One such mechanism could be that low doses affect a particular mechanism of memory formation that only results in a slight impairment and that high doses affect different mechanisms that are more critical to memory formation and thus, the deficits are more severe. However it seems more likely and other groups have shown that low and high doses of ethanol disrupt the same mechanisms of memory formation but at low doses there is not a high enough internal tissue concentration to completely disrupt these mechanisms.

Examination of the effects of ethanol on short-term habituation revealed some very interesting and important results as well. A large amount of research has been done on the effects of ethanol on short-term memory in many different animal models and in humans. However, there still remains a large discrepancy in the results from much of this research mostly due to the temporal pattern of administration of ethanol and when retention of short-term memory is tested. I chose the to study how ethanol affects short-term habituation at two separate ISIs to show that ethanol preferentially affects memory depending on the temporal pattern in which stimuli are administered. My finding that ethanol exposure specifically interferes with short-term memory when stimuli are given at longer ISIs but not at shorter ISIs highlights the importance of testing different
temporal patterns of stimuli presentation when investigating the effects of ethanol on short-term memory. Further, my results parallel the findings in human studies that there is less memory impairment when there is a short interval between the stimuli presentation and when subjects are asked to recall that stimuli (Acheson, et al., 1998; Nordby et al., 1999). Studies of short-term habituation in the crab *Chasmagnathus* did not find an impairment of short-term habituation to a visual stimulus when the crabs were exposed to ethanol, however during each trial the stimuli were presented at a 2s ISI (Saraco and Maldonado, 1995). This study suggests that ethanol exposure impairs habituation and short-term memory when subjects are tested at longer ISIs, therefore we hypothesize that ethanol would affect habituation in the crabs at a longer ISI.

Studies of short-term habituation to tap stimuli in *C. elegans*, and in all species studied to date, have revealed that habituation to short ISIs occurs at a faster rate and is more complete than habituation to stimuli delivered at long ISIs (Rankin and Broster, 1992). This is frequency dependence of habituation that was described by Groves and Thompson (1970). Research on habituation in *C. elegans* has led to the hypothesis that there are multiple mechanisms that underlie this form of learning and that the frequency of stimulus administration (i.e. the length of ISI) stimulates different molecular mechanisms (Rankin and Broster, 1992; Rose and Rankin, 2001). For this reason we tested the effects of ethanol on short-term habituation at both a short (10s) and long (60s) ISIs. Interestingly, we found that ethanol primarily alters habituation at long ISIs but not at short ISIs. This would suggest that the mechanisms that are responsible for habituation to lower frequency stimuli are disrupted by ethanol exposure but the mechanisms that are responsible for habituation at higher frequencies are resistant to the effects of ethanol. To
our knowledge this is the first report of a pharmacological manipulation that dissociates habituation *in vivo* into separate processes depending on the frequency at which stimuli are administered.

Though ethanol exposure has been shown to have depressive effects on behaviors such as locomotion and egg-laying in *C. elegans*, we see an opposite effect on behavior in our paradigm. When worms are exposed to a 0.4M external ethanol concentration their movements become uncoordinated and their locomotion is slowed. A logical prediction is that ethanol exposure would result in other behaviors being depressed. However, worms exposed to ethanol continue to respond with large responses to tap for longer than worms not exposed to ethanol. This suggests that the mechanisms that mediate the effects of ethanol on locomotion and egg-laying behaviors (i.e. the potentiation of SLO-1 by ethanol) may not be involved in habituation or may function differently.

One of the critical experiments detailed in this thesis was the experiment involving *avr-14*. Because I showed that short-term habituation was impaired when stimuli were given at a 60s ISI, it was important to investigate whether the impairment in the formation of long-term memory was due to a learning deficit as seen by the impairment in STH or whether ethanol also acted to impair the formation of memory through a different set of mechanisms. In this experiment we showed that the effect on STH at a 60s ISI is dissociable from the impairment of the formation of LTM of habituation training. Using the *avr-14* mutant, which exhibits LTM when training is administered at a 10s ISI, a frequency of stimuli administration that is relatively unaffected by ethanol, we found that ethanol impaired memory formation at both ISIs.
This result was very important because it indicated that alcohol has two different effects, one of which impairs short-term memory and one impairs long-term memory.

Researchers have recently shown that *C. elegans* is capable of ethanol-induced state-dependent learning in an olfactory adaptation assay (Bettinger and McIntire, 2004) as well as context-dependent memory for habituation training (Rankin, 2000). To identify if state-dependency or context conditioning was playing a role in our paradigm worms were trained in an intoxicating dose of ethanol and tested in a similarly intoxicating dose or a dose that would remind them of the ethanol context in which they were trained. The result that no memory was observed when a low dose of ethanol was present during testing shows that an ethanol context cannot produce memory supporting the idea that no memory was present to begin with. Further, our result that worms did not show any retention for memory when tested in an intoxicating dose of ethanol indicates that state-dependent learning does not play a role in the long-term memory for habituation training. A key difference between olfactory adaptation and habituation to tap is that worms that deficient in *glr-1*, a gene that is integral to long-term habituation to tap stimuli, did not show any abnormalities in the olfactory adaptation or state-dependency of olfactory adaptation. This suggests that different neuronal processes may mediate olfactory adaptation and LTM for habituation to tap and that ethanol may effect these processes in different ways.

In this study we found that increased tolerance to ethanol did not rescue the LTM deficits due to ethanol exposure. This was very interestingly for two major reasons. One is that previous studies that have looked at the behavioral effect of ethanol on *C. elegans* have shown that mutations that result in increased tolerance (*npr-1*) partially rescued
locomotory speed, however, in this study these same mutations do not rescue LTH suggesting that the effects of ethanol on memory formation may be distinct from the effects on other behaviors including locomotion. Second, the results indicate that tolerance to ethanol plays an important role in helping to recover from very simple behaviors but relatively more complex behaviors that involve more complex cellular mechanisms and signaling such as memory formation, may be independent of ethanol tolerance. However, Davies et al. (2004) only tested acute tolerance (<60 min after ethanol exposure) and it may be the case that npr-1 mutants show increased tolerance only during shorter time periods and these mutants may not have increased tolerance to ethanol for the length of ethanol exposure in our LTH paradigm (7 hrs). Further testing and investigation into this hypothesis will be required.

Further, we found that the formation of LTM was inhibited only when worms were exposed to ethanol during, but not after habituation training. This is an important finding because it shows that ethanol has a specific effect on the formation of memory because it does not disrupt the previously learned information. Similarly, Beck & Rankin (1995) found that heat-shock disrupted the formation of LTM for habituation training only during the training period. They concluded that some of the mechanisms that are responsible for the induction of LTM occur during the training process. The cellular response to heat-shock is the termination of all other proteins other than heat-shock proteins, which indicates that the induction of long-term memory for habituation requires protein synthesis. My data indicate that exposure to ethanol has a similar effect to heat-shock by blocking the induction of LTM. Though I do not suggest that ethanol exposure blocks protein synthesis, I do suggest that ethanol exposure interferes with some of the
same mechanisms that are initiated during habituation training to induce LTM. More recently, Rose et al. (2003) showed that the activation of non-NMDA-type glutamate receptors during the training period is required for the induction of LTH. This result led me to hypothesize that ethanol may be interfering with glutamatergic neurotransmission during the training period.
Chapter II: Ethanol Exposure Interferes with GLR-1 Regulation

II.1 Ethanol Exposure Increases GLR-1::GFP

A number of researchers have shown that in rodents one of the most likely mechanisms that underlie memory involves the trafficking of AMPA-type receptors following LTP/LTD-like stimulation (Malenka and Bear, 2004). Interestingly, the expression and trafficking of a number of different glutamate receptor types is altered by exposure to ethanol (Chandler et al., 1999; Chandler, 2003; Sheela Rani and Ticku, 2006). Using *C. elegans*, Kwon et al. (2004) showed that high doses of ethanol can rapidly induce the expression of *glr-2*, a homolog to the mammalian GluR2 AMPA receptor subunit. To identify whether a similar phenomenon was occurring in our paradigm I tested whether ethanol exposure would cause an increase in *glr-1*. I wanted to investigate changes in *glr-1* rather than *glr-2* for two main reasons. First, the expression pattern of *glr-2* in the tap-withdrawal circuit is difficult to observe because of very low expression levels. Second, *glr-1*, which encodes an AMPA/KA-type glutamate receptor subunit, has been shown to be critical for the induction and expression of LTM for habituation training in *C. elegans* (Rose et al., 2003). Using a reporter gene that has GLR-1 tagged with a green fluorescent protein (GLR-1::GFP), I examined whether ethanol exposure would result in any changes in the expression of GLR-1. To accomplish this, worms were exposed to ethanol for 7 hrs (the same amount of time need to complete habituation training) and imaged with a confocal microscope 1 hr following exposure.
Figure 15. Ethanol Exposure results in increased levels of GLR-1::GFP. Comparison of the naïve and ethanol treated worms reveals that ethanol treated worms have significantly higher levels of GLR-1::GFP than naïve groups.

An overall ANOVA revealed a significant effect of exposure, which showed that the ethanol group had higher GLR-1::GFP expression than the non-ethanol group ($F_{(1,43)}=4.941, p<0.05$; Fig 23). This result shows that ethanol exposure resulted in an increase in the expression of this ionotropic glutamate receptor subunit.

II.2 Ethanol Disrupts the Decrease in GLR-1::GFP Normally Associated with Long-term Habituation

The expression of LTM for habituation training is correlated with a down-regulation of GLR-1, as measured using a green fluorescent protein (GFP) fusion to GLR-1 (GLR-1::GFP; Rose et al., 2003). To investigate whether the increase GLR-1::GFP following ethanol exposure interferes with the normal down regulation of GLR-1::GFP seen after habituation training, mutant worms carrying the GLR-1::GFP construct were exposed to ethanol, administered habituation training and the expression of GLR-1::GFP was examined 24 h later using confocal imaging. An overall ANOVA revealed a
significant effect of training and ethanol exposure on the total area of GLR-1::GFP observed (F (3,64)=5.179, p<0.01). A comparison of the total amount of GFP in the non-ethanol groups showed that the trained animals had significantly lower levels of GLR-1::GFP than the control group (p<0.05; Fig 16), which is consistent with previous published data (Rose et al., 2003).

Figure 16. Ethanol Exposure During Training Interferes with Training-Induced Decreases in GLR-1::GFP. In the naïve condition, trained worms show a decrease in total amount of GLR-1::GFP 24 hrs after habituation training in the ventral nerve cord. The total area of GLR-1::GFP observed in worms trained in the presence of ethanol does not differ significantly from either ethanol-exposed control or naïve control worms.

However, in the ethanol treated groups we found that there was no significant difference between the total levels of GFP between trained and control groups (p>0.05; Fig 24). We also observed that there was a significant difference between the ethanol and naïve control groups in GLR-1::GFP expression (p<0.05). It is important to note that the standard error of the ethanol exposed groups are larger than the naïve groups, which is indicative of the variability of ethanol’s effects on GLR-1::GFP levels. This result reveals that under control conditions ethanol exposure causes a long-lasting increase in GLR-1::GFP but if habituation training and ethanol are administered at the same time,
this increase is blocked. Interestingly, this suggests that there may be an interaction between habituation training and ethanol exposure on the regulation of GLR-1::GFP. This result confirms that ethanol exposure disrupted the decrease in GLR-1::GFP that is required for the formation of LTM.

Discussion Chapter II

There is a large body of research discussing the importance of glutamatergic neurotransmission in response to ethanol treatment in a number of different paradigms and species. My results are consistent with this in that I show that ethanol exposure increases the expression level of GLR-1::GFP along the ventral nerve cord in *C. elegans*. Further, even 24 hrs after ethanol exposure, I observe a significant increase in the amount of GLR-1::GFP between ethanol-treated and naïve groups. This result suggests that ethanol exposure induces a change in glutamate receptor subunit expression and this change is long-lasting. Previous research has also shown that the transcriptional level of another AMPA/KA-type glutamate receptor subunit, *glr-2*, is rapidly induced following exposure to high concentrations of ethanol (Kwon et al., 2004). This suggests that the increase in GLR-1 expression that we observed is due to changes in the transcriptional regulation of *glr-1*. This hypothesis would be easily tested by examining mRNA levels following ethanol exposure using quantitative RT-PCR following ethanol exposure. This idea is further supported by work done using mammalian neuronal cultures that has shown that expression of specific ionotropic glutamate receptor subunit mRNAs are increased in responses to ethanol treatment under certain environmental conditions (Hu et al., 1996). Further, research done *in vivo* has revealed increases in the protein levels of the NMDA receptor subunits in response to a number of different ethanol exposure
paradigms (Follesa and Ticku, 1995; Snell et al., 1996; Sircar, 2006). However, to our knowledge, our study is the first to show increases in the protein level of an AMPA/KA-type glutamate receptor subunit in vivo.

In the imaging studies I found that compared to naïve control worms, ethanol exposed control worms had significantly higher levels of GLR-1::GFP yet the ethanol exposed trained worms did not differ significantly from naïve control worms. This finding highlights the fact that if exposed to ethanol for 7 hrs, worms still show a significantly higher level of GLR-1::GFP 24 hrs later, indicating that there is a long-lasting effect of ethanol exposure in the nervous system of these worms. The result that habituation training and ethanol exposure have a significant and long-lasting effect on the levels of GLR-1::GFP suggests that the molecular mechanisms that mediate training-induced decreases in GLR-1::GFP may have similar components to the molecular pathways that mediate ethanol-induced increases in GLR-1::GFP. Further research is needed to identify which signaling pathways result in the decreased level of GLR-1::GFP in trained animals and whether similar or different pathways are effected by ethanol exposure.

In the final experiment of this chapter we found that 24 hrs after habituation training, the ethanol exposed groups did not differ significantly in the total area of GLR-1::GFP observed, whereas the naïve group did with the trained group having significantly lower GLR-1::GFP. This pattern is the same as for the behavior observed at the same time point; the ethanol treated groups do not differ in their response to tap and the untreated trained group shows significantly smaller responses than the naïve control group. This highlights the importance of GLR-1 in mediating the expression of LTH
because like previous studies have shown, pharmacological manipulations that block LTH also do not result in a difference in the amount of GLR-1::GFP 24 hrs later.

A number of other groups have investigated how ethanol administration affects the expression of glutamate receptor subunits in mammals and have found that ethanol seems to primarily affect the NR2B subunit. In our assay we have only thus far assessed the role GLR-1 in response to ethanol and have found a significant effect. This does not rule out the possibility that the NMR-2 subunit (the *C. elegans* homologue to NR2B) will play a role and I hypothesize that it will as well. Interestingly, in some ways the AMPA/KA-type receptors in *C. elegans* are similar in function to mammalian NMDA receptors. For example, both *C. elegans* AMPA/KA-type receptors and mammalian NMDA receptors are permeable to calcium (Zheng et al., 1998). This ability is a critical component to synaptic plasticity in mammalian systems and may be the reason why LTM for habituation is not dependent upon *nmr-1* but is dependent upon *glr-1* in *C. elegans*. This also suggests that in *C. elegans* AMPA/KA-type receptors may serve as the functional equivalent of both AMPA/KA-type receptors and NMDA receptors in mammals.

Immediate further research should focus on the mechanisms by which ethanol is regulating the expression of *glr-1* that results in the blockade of memory formation. A significant amount of research has focused on and revealed the importance of the glutamatergic post-synaptic density (PSD) as a target of ethanol (Chandler, 2003). I believe it will be of critical importance to uncover if ethanol is acting at glutamatergic PSDs to increase levels of GLR-1 in *C. elegans*. However, most of the research that has been done so far elucidating how ethanol effects receptor complexes and signaling
cascades has been done using cell culture and *in vitro* methods. It is important to note that at a systems level, the overall effect of ethanol is to depress the nervous system and as a compensatory mechanism, excitatory, glutamatergic neurotransmission may be up-regulated. Thus, it will also be very important to investigate whether genes or mechanisms that regulate the excitatory to inhibitory balance in the nervous system will be affected by ethanol exposure.
GENERAL DISCUSSION

The goal of this thesis was to assess the effect of ethanol on memory processing in *C. elegans* in order to increase our understanding of this process and to gain further insight into the molecular mechanisms that underlie these effects. The experiments outlined in this thesis offer a new model in which to study the effects of ethanol on both short- and long-term memory.

I found that ethanol exposure did not alter rates of habituation at short ISIs (10sec) however; habituation was significantly slowed when habituation occurs at longer ISIs (60sec). The differential effect that we observed in ethanol exposed lends support to the hypothesis that was first proposed by Rankin and Broster (1992) stating that habituation is not mediated by a singular mechanism but that it is mediated by a number of different molecular mechanisms. It appears that the cellular mechanisms that underlie habituation are differentially recruited depending on the stimulus frequency or ISI. Interestingly, this result parallels the finding in mammalian electrophysiology that if one stimulates the CA1 region of the hippocampus 4 times at a relatively high frequency (100Hz), LTP can be observed. However, if one stimulates the same region at a low frequency (1Hz), then a completely different phenomenon, LTD, is observed. Further depending on the protocol being used and brain region being studied to induce LTP or LTD, the effects can be short or long-lasting and may require different molecular mechanisms (Malenka and Bear, 2004).

In my experiments I showed that ethanol exposure during the habituation training significantly impaired the formation of LTM. The behavioral effects of ethanol exposure in *C. elegans* have been assessed in a number of different behavioral paradigms,
including locomotion, egg-laying, and chemotaxic adaptation. The work presented here also shows that ethanol has a significant effect on long-term memory in *C. elegans*, which has striking similarities to ethanol-induced memory deficits observed in a number of other species.

In the final chapter, I found that the regulation of *glr-1* expression was impacted by ethanol exposure and may be an important mechanism for ethanol's disruption of memory formation. When I trained worms in the presence of ethanol I did not observe a decrease in the expression of GLR-1::GFP, a cellular correlate of long-term memory for habituation training. However, I did observe a significant increase in GLR-1::GFP immediately after 7 hrs of exposure to ethanol showing that ethanol exposure results in alterations in the post-synaptic density.

Using *C. elegans* to identify ethanol-induced memory deficits and the molecular mechanisms underlying them has led to a number of important discoveries. These experiments have identified a novel model system in which to study ethanol-induced memory deficits, have identified differential effects of ethanol on STH (depending on the ISI), further supported the importance of GLR-1 in LTH and in response to ethanol exposure. This research will inevitably lead to new insights not only into how ethanol effects memory processing but also to the discovery of molecular mechanisms that underlie normal learning and memory.

**Limitations and Future Direction**

The research detailed above has led to the identification of a novel model in which to study the negative impact on the memory processing and has started to uncover some of the mechanisms by which this is occurring. However, there are some limitations
with the generalizability of this research to higher order organisms including rodents and humans. First, the simplicity of the nervous system of *C. elegans* allows it to be very tenable for molecular and genetic manipulation however, this simplicity only allows for the identification and measure of very simple behaviors including habituation. Also, it becomes more difficult to generalize some of the cellular actions of ethanol to higher order species because many groups have found that the actions of ethanol are highly dependent on the type of neurons and cortical locations that are being studied.

Much of the research into the behavioral consequences of ethanol exposure has been split into two major genres: acute exposure and chronic exposure. In rodent models acute exposure has mainly been injection or ingestion of known concentrations of ethanol immediately prior to testing while chronic testing has been administration of ethanol over longer periods of time from weeks to months of daily doses of ethanol. Due to the relatively short lifespan of *C. elegans*, it becomes much more difficult to identify whether the paradigms we are using are acute or chronic exposures. In relation to ethanol exposure during STH, it can be stated with relative confidence that our paradigm is acute exposure however it becomes a bit more complex when we look at the LTH ethanol exposure paradigm. Because the ethanol exposure is 6hrs 20min, we would assume that is chronic because that is a significant part of *C. elegans* lifespan (which is only 14-21 days) but what we do not know is whether the temporal administration of ethanol has a similar effect on the nervous system of different species regardless of lifespan or whether the detrimental effects of ethanol exposure are a function of lifespan.

Another limitation of this study is that we have only begun to scratch the surface of the cellular effects of ethanol that impair memory formation. It will be very important
to further identify parallels with ethanol-related mammalian research including identification of any changes in NMDA receptor and GABA receptor homologues. However, because *C. elegans* is a very simple organism with less than 1000 cells, it is difficult to test mutations in some of the specific genes that may play a role in ethanol-related signaling pathways because these genes are present in many different cell types in the worm, which sometimes leads to unviable or un-testable mutations. Also, some of the more complex signaling pathways that are affected by ethanol in mammals that may also effect memory functioning do not have homologues in *C. elegans*.

Finally, the behavioral effects of ethanol exposure in mammals are highly dependent on a number of factors with one of the major factors being individual differences in susceptibility. Testing for the effects of ethanol on memory processing in a clonal population, like we have done may only allow us to partly observe the effects that we would see in a population in the wild. We also know that the behavioral effects of ethanol treatment can be mediated by its effect on other tissues other than the nervous system. In other systems and preparations researchers can inject ethanol directly into different areas of the brain and observe any resulting effects, thereby bypassing the effect of ethanol on other tissues, something that is technically impossible to achieve in *C. elegans*. Therefore, one of the limitations that we have in our system is that some of the effects we observe may in part be mediated by an unknown effect on other tissues or systems in this species.

Despite these constraints *C. elegans* offers unique opportunities to study the impact of ethanol on learning and memory. The ethanol-induced memory impairments that I observed in this model system show a number of similarities to those observed in
other species including rodents and humans. Through the use of *C. elegans* we have shown that ethanol disrupts specific mechanisms that impair some but not all learning and memory processes. The high degree of genetic tractability and molecular manipulation of *C. elegans* allows for relative ease in uncovering the mechanisms that underlie these deficits. Further analysis using this model will allow for the identification of key cellular mechanisms that underlie learning impairments and deficits in memory formation as a result of alcohol consumption. Similarly, the identification of genes that are important in mediating the effects of alcohol will also have important consequences in determining susceptibility to alcohol-induced behaviors and alcohol-related disorders.
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