CORRELATED CHANGES IN BEHAVIOUR AND GLUTAMATE RECEPTOR EXPRESSION AS A RESULT OF EARLY STIMULATION IN CAENORHABDITIS ELEGANS

By

Celia Mariah Ebrahimi

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ABSTRACT

The effects of early sensory experience on the development of central nervous system structures and behavior are the focus of much ongoing research. Previous studies have demonstrated that exposing animals to sensory enriched environments produced significant changes in the nervous system and accelerated and improved the development of complex cognitive behaviors such as learning and memory; however the long-term effects of sensory enriched environments on the nervous system and behavior remain unknown. In this study, I investigated the long-term effects that an enhancement in mechanosensory experience produced on adult behavior and glutamate receptor-1 (GLR-1) distribution in the nematode Caenorhabditis elegans (C. elegans). I found that C. elegans exposed to an enhancement in mechanosensory stimulation exhibited different behavioral and molecular outcomes depending on the temporal pattern of mechanosensory stimulation, the age of the worm stimulated and the age of the worm tested. Early spaced mechanosensory stimulation at a long inter-stimulus interval produced two independent behavioral effects, an early enhancement at 3 days followed by a depression at 5 days. Both behaviors were dependent on glutamate activity and were associated with a positively correlating change in both glutamate receptor-1 protein distribution and mRNA levels. The 3 day behavioral enhancement was not observed when spaced mechanosensory stimulation was delivered later in larval development, suggesting a critical period for these effects early in larval development. There appeared to be no critical period for the depressed behavioral response observed at 5 days; however only the depressed behavioral response of 5 day old worms was found to be sensitive to reconsolidation blockade. These results suggest that early mechanosensory stimulation produces two independent effects in adult worms: an early developmental effect in 3 day old worms which is mediated by one
cellular event and a long-term memory effect at 5 days which is mediated by a separate cellular phenomenon. The results from this study have provided novel information into the long-term effects that an enhancement in early sensory experience had on the behavior and molecular properties of the adult nervous system, and provided the foundation for future research in this field.
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INTRODUCTION

The biological and psychological development of the central nervous system is known to be highly dependent on pre and postnatal interactions between an organism and its environment. These environmental interactions critically regulate both positive and negative effects on the synaptic development of nervous system structures and their correlated behaviors (Grossman et al., 2003). Studies using humans have shown that postnatal sensory experiences beginning in the first months of life and extending into the eighth year can have significant effects on the development of adult behaviors and intellect (Ramey, Bryant and Suarez, 1985; Greenough, Black and Wallace, 1987; Ramey and Ramey, 1992). However, studies exploring the effects of early sensory experience on the development of the human nervous system are limited to clinical trials and thus it is not possible to control all of the variables at work. Therefore, to gain insight into the biological effects that early experience has on structures within the brain, research is conducted using animal model systems.

Research investigating the effects of early experience on the development of the central nervous system and behavior are conducted using one of two experimental paradigms (Hebb, 1949; Turkewitz and Kenny, 1982; Rosenzweig and Bennett, 1996). The first paradigm involves the early deprivation of certain classes of sensory stimulation (i.e. visual, auditory or mechanical stimulation). These studies assume that a developing organism requires the presence of specific stimulation at particular developmental times for wild-type nervous system and behavioral development, and that the absence of such stimulation will impair wild-type development. This has been shown experimentally in numerous studies that have looked at the effects of sensory deprivation on the behavior and properties of the nervous system using both vertebrate and invertebrate model systems (Diamond et al., 1966). Rodents reared in sensory
deprived environments have thinner visual cortices (Volkmar and Greenough, 1972), decreased body and brain weight (Rosenzweig, Love and Bennett, 1968), reduced synaptic contacts per neuron (Turner and Greenough, 1985), decreased performance in spatial learning tasks and poorer motor coordination compared to rodents reared in standard laboratory conditions (Turner and Greenough, 1985). The second experimental paradigm involves an early enrichment of environmental stimulation (Hebb, 1947). In this type of environment animals experience a combination of complex inanimate and social stimulation (van Praag, Kempermann, Gage, 2000). In general enriched animals are kept in larger cages and in larger groups with more opportunity for more social interaction and physical activity. In addition, the environment is complex (i.e. novel toys, running wheel) and is varied over the period of the experiment (Hebb, 1947; van Praag et al., 2000). Studies investigating the effects of environmental enrichment in rodents have shown that rodents reared in enriched environments exhibited an increase in total brain weight (Rosenzweig et al., 1968; Wainwright et al., 1993), total cortical weight (Rosenzweig, Bennett and Diamond, 1972), cerebral cortex thickness (Uylings, Kuypers, Diamond and Veltman, 1978), the density of hippocampal neurons (Kempermann, Kuhn and Gage, 1997), synaptic contacts (Walsh and Cummins, 1976), the number of dendritic branches (Greenough and Volkmar, 1973; Rosenzweig and Bennett, 1996) and mRNA levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptor 2 and 4 (Naka, Narita, Okado, and Masaake, 2005) compared to rodents reared in standard laboratory conditions. In addition, environmental enriched reared rats show improved spatial learning (Morgensen, 1991; Janus, Koperwas, Janus and Roder, 1995) motor learning (Whishaw, Zaborowski, Kolb, 1984; Tees, Buhrmann and Hanley 1990; Fernandez-Teruel et al., 1997) and a precocial development of the visual system (Cancedda et al., 2004; Dumas, 2004) compared to rats reared in standard laboratory conditions. However, a major
problem to investigators looking at the effects of environmental enrichment on the development
of the nervous system is that the relevance of single contributing environmental factors cannot
be easily distinguished. Thus, the importance of specific types of stimulation are difficult to
assess. This problem is being addressed by several investigators comparing whether the
enhancement of a single behavioral task experienced in an enriched environment (i.e. physical
activity) produces changes in the nervous system comparable to those observed in animals
reared in enriched or standard laboratory environments. Similar to enriched reared animals,
rodents given voluntary access to a running wheel exhibited a significant increase in the rate of
neurogenesis in the dentate gyrus (van Praag, Kempermann and Gage, 1999), improved
performance in spatial learning and reference memory tasks (Rozenweig and Bennett, 1996;
Kempermann et al., 1997; van Praag et al., 1999), and significantly more short-term and long-
term potentiation (i.e. cellular models of synaptic plasticity; Farmer et al., 2004), compared to
rodents reared in standard laboratory conditions. In addition, the enhanced experience of a
single sensory variable has been shown to produce significant effects on the development of
the nervous system and behavior. Dumas (2004) demonstrated that suturing open the eyes of
rat pups two days prior to normal eye lid opening resulted in the accelerated development of
the hippocampus and hippocampal-dependent behavior (i.e. spontaneous alternation). Thus,
the enhancement of a single behavioral task or sensory variable above and beyond what an
organism normally experiences is sufficient to produce significant changes in the nervous
system that resemble those observed in enriched reared animals. These studies clearly
illustrate that an enhancement of early sensory experiences significantly alters the wild-type
development of central nervous system structures and behavior.

Despite the number of studies investigating the effects of early sensory experience on
the development of the central nervous system and behavior, very few studies have explored
whether these changes are prolonged into adulthood. The majority of studies conducted have investigated the short term effects (three to four weeks) of early experience on the development of the vertebrate central nervous system structures and behavior (Briones, Klintsova and Greenough, 2004); however whether these effects continue into adulthood or if there is a cost to the precocial development of the nervous system and behavior is unclear. A number of factors influence the amount of information available on the long-term effects of early sensory experience on the vertebrate nervous system. First, due to the complexity of the vertebrate nervous system and its development, it is difficult to isolate the effects that early experience has on the development of the nervous system. Second, the effects of maternal behavior (licking, grooming and nursing style) have been shown to influence normal development (Fernandez-Teruel et al., 1997; Pham, Sodertrom, Henriksson and Mohammad, 1997; Meaney, 2001; Liu et al., 2002). Third, vertebrate development can occur over a period of years resulting in great difficulty in controlling all environmental variables. One solution to these problems is to investigate the long-term effects of sensory enhancement using a simpler model system with a shorter developmental timeline.

Prior to 1985, most of the research investigating the effects of early experience on an animals central nervous system and behavior were conducted primarily using mammalian model systems. This was due to a common misperception that the invertebrate nervous system was rigidly genetically pre-programmed during development and was therefore considered to be qualitatively different from the vertebrate nervous system, which was believed to exhibit a higher degree of developmental plasticity in response to early experience. However, recent studies have recognized the biological and psychological effects early experience has on the invertebrate nervous system causing a re-evaluation of this view. Punzo and Ludwig (2002) showed that the early experience of Hogna carolinensis (Lycosidae) spiderlings with the
maternal parent and siblings produced a significant increase in the protocerebral neuropil and influenced spatial learning. *Acheta domesticus* (crickets) exposed to sensory deprived environments during early life contain less neurons when compared to crickets raised in sensory enhanced environments (Lamoesse et al., 2000). Barth et al. (1997) demonstrated a significant decrease in the size of the optic lobes in monocularly deprived *Drosophila melanogaster*. Finally, Rose et al. (2005) found that adult *Caenorhabditis elegans* (*C. elegans*) raised in isolation exhibited significantly smaller reversal behavioral responses to a mechanical tap stimulus, were significantly smaller in size and had smaller glutamatergic synapses along their posterior ventral nerve cord compared to worms raised in colonies. These studies demonstrate the presence of activity dependent plasticity in animal model systems with short developmental lifespans, and suggest possible animal model systems to investigate the long-term effects of early sensory enhancement.

**CAENORHABDITIS ELEGANS AS A MODEL SYSTEM**

The self fertilizing hermaphrodite *C. elegans* was selected by Sydney Brenner as an ideal model system to investigate the properties of the nervous system and the process of development (Brenner, 1974). The adult worm is made up of 959 cells, 302 of which are dedicated to forming the nervous system. The cell lineage and anatomical location of all 302 neurons and the synaptic relationships between neurons is well characterized (White, Southgate, Thomson and Brenner, 1986; Chalfie et al., 1985). Despite the simplicity of the *C. elegans* nervous system, many of the receptors, receptor trafficking proteins, and neurotransmitters used by this organism are similar to those found in mammalian model systems. In addition, the nervous system of the worm is comprised of sensory neurons, interneurons and motor neurons; however, unlike mammalian model systems, the worm can
remain viable despite catastrophic mutations to the nervous system (Hedgecock, Culotti, Hall and Stern, 1987). Furthermore, the transparency of this organism permits investigators the ability to observe and quantify neural protein expression in an intact animal using marker constructs such as green fluorescent protein (Chalifie et al., 1994). Thus, the properties of the C. elegans nervous system provide investigators with an ideal model system to explore environmental and genetic factors regulating neural development and activity dependent plasticity.

The short lifespan of C. elegans, complete developmental lineages for every cell type in the adult, and ease of obtaining large populations of worms presents researchers with an ideal model system for developmental investigations (Sulston and Horvitz, 1977; Sulston, Schierenberg, White and Thomson, 1983). The worm hatches from its egg at approximately 12 hours post egg-lay (at 20 degrees centigrade) into the first of four larval stages (L1, L2, L3 and L4; Figure 1). At hatching the worm is capable of swimming on the agar surface in a similar sinusoidal pattern similar to that seen in adult worms (Croll, 1975). At approximately 70 hours post egg-lay (at 20 degrees centigrade) the worm reaches sexual maturity and begins to lay eggs (Davis, pers comm.). A worm will lay approximately 250 – 350 eggs during its lifetime, and the parental worm does not need to deliver any form of maternal stimulation to its offspring progeny in order for normal development to occur. The absence of maternal behaviors allows researchers the ability to explore the effects of mechanical stimulation alone on the molecular properties and function of the C. elegans nervous system. The worm can survive under ideal temperature and food conditions for approximately 17 days after adulthood (Riddle, Blumenthal, Meyer and Priess, 1997). Thus, the short developmental timeline and the absence of maternal stimulation make C. elegans an ideal model system to explore the long-term effects of sensory enhancement on the development of the nervous system and behavior.
**Developmental Stage**

<table>
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<tr>
<th>Stage</th>
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<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>3 Days</th>
<th>4 Days</th>
<th>5 Days</th>
</tr>
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<tr>
<td>Hours (20°C)</td>
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<td>.12</td>
<td>26</td>
<td>36</td>
<td>45</td>
<td>60</td>
<td>84</td>
<td>108</td>
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**Figure 1. C. elegans Developmental Timeline.** The typical life cycle of *C. elegans* in hours at 20 degrees centigrade. Eggs are laid approximately three hours after fertilization: 0 represents the time at which eggs are laid. *C. elegans* hatches from the egg at approximately 12 hours after egg-lay into the first of four larval stages (L1 – L4). Animals spend approximately 2.5 days in larval development (including embryogenesis), and begin to lay eggs at 3 – 3.5 days.

**CAENORHABDITIS ELEGANS TAP WITHDRAWAL CIRCUIT**

Locomotion in *C. elegans* begins as soon as the worm hatches from the egg and is characterized by sinusoidal waves propagating down the length of the worm’s body as it swims over or through agar filled Petrie dishes. The application of a gentle touch to the head or tail of the worm results in a backward or forward swimming response respectively. These responses are not specifically localized to the head or tail. A general mechanical tap delivered to the side of the Petrie dish the worm inhabits will result in a backward swimming response (Rankin et al., 1990). The magnitude of this reversal response is termed the tap withdrawal reflex and is the primary behavior focused in the experiments reported in this thesis.

The neural circuit mediating the tap withdrawal reflex has been functionally, developmentally and genetically described. The primary elements of the tap withdrawal circuit are the five touch receptors and four pairs of command interneurons (Wicks and Rankin, 1995). The tap withdrawal circuit was shown to be comprised of two sub-circuits. The first sub circuit produced forward movement and was mediated by the touch cells (ALML/R, AVML/R) in the head and two pairs of command interneurons (AVA and AVD). The second sub circuit produced reverse movement and was mediated by touch cells in the tail (PLML/R) and two pairs of command interneurons (AVB and PVC). Both PVD (touch cell) and DVA...
(command interneuron) appear to be involved in integrating the competing inputs from the head and tail (Wicks and Rankin, 1995). The delivery of a mechanical tap stimulus to the side of the Petrie dish containing the worm simultaneously activates both sub-circuits, and produces a reversal response. This reversal response is mediated by electrical gap junctions from the touch cells onto the interneuron’s of the reversal sub-circuit and chemical connections from touch cells onto the interneurons of the forward sub circuit. Wicks and Rankin (1997) also showed through laser ablation studies, that the chemical synapses from the touch cells onto the interneuron’s in the tap withdrawal circuit were the most likely sites of synaptic plasticity. This information permits for the genetic dissection of short-term and long-term habituation to the tap stimulus in *C. elegans*.

**Figure 2. Tap Withdrawal Response Neural Circuit.** Rectangles represent the touch cells, circles represent the interneuron’s that connect onto the triangles representing the motor neuron pools. Dashed lines represent gap junctions (electrical connections) while solid lines represent chemical connections. Dark grey neurons (PLM, AVB and PVC) mediate forward locomotion, while medium grey neurons (ALM, AVM, AVA and AVD) mediate reversal locomotion (swimming backwards). It has been proposed that the tap stimulus simultaneously 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).
During *C. elegans* post-embryonic development a number of biological changes take place in the tap withdrawal sub circuit that is responsible for mediating the reversal response to tap. At hatching the L1 the worm locomotes in a manner similar to adults suggesting a functional tap withdrawal circuit (Chalfie et al., 1985); however, there are significant differences between the structure of the larval and adult tap withdrawal circuits. The newly hatched L1 larva has only four lateral touch receptor cells, AVM and PVM arise during L1 at approximately 11.5 hours after egg lay (at 20 degrees centigrade; Chalfie et al., 1985) and PVD arises during L2 at approximately 22.5 hours after egg lay (at 20 degrees centigrade; Driscoll and Kaplan, 1997). During L1 and L2, both pairs of ALM touch receptor cells work independently via the AVD interneurons. By approximately 32.5 to 37.5 hours after egg lay (at 20 degrees centigrade), AVM connects to ALM cells and provides additional outputs to the AVD cells and a unique output to the AVB interneurons (Chalife et al., 1985; Driscoll and Kaplan, 1997). The addition of AVM to the tap withdrawal circuit during L3 and the birth of PVD during L2 have been hypothesized to account for the different tap withdrawal behaviors seen in larval and adult worms (Driscoll and Kaplan, 1997). Larval worms exhibit an equal probability of swimming backwards or forwards in response to tap; however the additional output provided by AVM introduces a bias towards a reversal response to the tap stimulus in adult worms (Broster and Rankin, 1994). In addition to the functional wiring of AVM to the tap withdrawal circuit during L3 and the birth of PVD during L2 56 motor neurons or glial cells are added to the ventral nervous system (Driscoll and Kaplan, 1997). This includes the AS cells which receives the major chemical output of AVB (Chalfie et al., 1985). Thus, the tap withdrawal circuit undergoes a number of post-embryonic biological changes that have been shown to significantly affect the tap withdrawal behavior of *C. elegans* during development.
HABITUATION IN *CAENORHABDITIS ELEGANS*

Habituation is defined as a progressive decrease in the vigor of an elicited response as a result of repeated presentation of the eliciting stimulus (Groves and Thompson, 1970), and is considered to be the simplest form of learning as it has been demonstrated in almost all organisms including single-celled protozoa (Wood, 1988). *C. elegans* has been shown to exhibit habituation to the mechanical tap stimulus in ideal temperature and humidity environments. Worms exposed to the repeated application of a mechanical tap stimulus delivered at a 10 second, 30 second, 60 second, or 90 second inter-stimulus interval exhibited a progressive decrease in the magnitude of the tap withdrawal reflex with each successive tap (Rankin, Beck and Chiba 1990; Tan, pers comm.). Thus, similar to other organisms, *C. elegans* are capable of exhibiting short-term habituation to the repeated presentation of an irrelevant stimulus.

Adult *C. elegans* are capable of forming long-term habituation to the mechanical tap stimulus. Worms subjected to a distributed habituation training paradigm [4 blocks of 20 taps delivered at a 60 second inter-stimulus interval with each block followed by a one hour rest period] 24 hours prior to testing [10 taps delivered at a 60 second inter-stimulus interval] showed a significant decrease in the magnitude of the tap withdrawal reflex across all 10 test taps in comparison to untrained control worms (Beck and Rankin, 1997; Rose, Kaun and Rankin, 2002). Some of the factors that were shown to influence long-term memory formation in *C. elegans* were protein synthesis and the pattern of stimulation delivered (i.e. massed versus spaced). Heat shock interrupts protein synthesis by producing heat shock proteins that function to prevent protein denaturation during periods of cellular stress (Lindquist, 1986). The administration of heat-shock during the one hour rest periods during habituation training abolished 24 hour retention to the tap stimulus in *C. elegans* demonstrating that long-term
memory in the worm was dependent on protein synthesis (Beck and Rankin, 1995; Rose et al., 2002). In addition, the delivery of spaced training (training trials presented with intervening rest intervals) has been shown to be more effective than massed training (the same number of training stimuli with no short intervening rest intervals) in producing long term memory for a variety of tasks in humans (Ebbinghaus, 1885), *Aplysia* (Carew, Pinsker and Kandel, 1972), rats (Fanselow and Tighe, 1988; Barela, 1999), *Drosophila* (Tully, Preat, Boynton and Del Vecchio, 1994), and *C. elegans* (Beck and Rankin, 1997; Rose et al., 2002). 24 hour retention in *C. elegans* was observed when stimuli were delivered in a spaced protocol and was not observed when training was delivered using a massed stimulation protocol (Beck and Rankin, 1997; Rose et al., 2002). Thus, similar to humans and other animal model systems, the formation of long-term memory in *C. elegans* was found to be dependent on protein synthesis and spaced stimulation.

**GLUTAMATE RECEPTORS**

- Glutamate is an excitatory neurotransmitter involved in rapid synaptic communication between neurons in both vertebrate and invertebrate nervous systems. Synaptic communication is mediated by a diverse population of ionotropic and metabotropic glutamate receptors formed by tetrameric or pentameric arrangement of subunits (Laube, Kuhse and Betz, 1998; Manom and Tiechberg, 1998; Rosenmund, Stern-Bach and Stevens, 1998). In vertebrates, 18 ionotropic glutamate receptor subunits have been identified, 7 NMDA (N-methyl-D-aspartate) type glutamate receptors and 11 non NMDA type glutamate receptors, which are further subdivided into AMPA and kainite subtypes (Brockie et al., 2001). In *C. elegans*, 10 putative ionotropic glutamate receptor subunits have been identified; eight non-NMDA receptor subunits (GLR-1 – GLR-8), and two NMDA receptor subunits (NMR-1 and
NMR-2; Hart, Sims and Kaplan, 1995; Brockie and Maricq, 2003; Brockie et al., 2001). In both vertebrate and invertebrate nervous systems, different combinations of AMPA type glutamate receptor subunits interact to generate a functionally diverse population of heteromeric glutamate receptor channels able to provide diverse functional properties such as learning and memory (Larson et al., 1995; Staubli et al., 1996; Hollmann, 1999, Morrison and van der Kooy, 2001).

Results from several studies investigating behaviors mediated by the tap withdrawal circuit (i.e. memory to tap, nose touch response) have suggested that the synapses onto the interneurons in the tap withdrawal circuit are glutamatergic, as mutations in one or more glutamate receptor subunit produced significant behavioral defects in wild type worms (Hart et al., 1995; Wicks and Rankin, 1997; Brockie et al., 2001; Morrison and van der Kooy, 2001; Mellem et al., 2002; Rose et al., 2002; Rose et al., 2003). In addition, Brockie et al. (2001) demonstrated that the tap withdrawal circuit interneurons coexpressed up to four non-NMDA subunits (GLR-1, GLR-2, GLR-4, and GLR-5). These subunits interact to form a diverse population of heteromeric glutamate receptor channels which mediate a number of different behaviors in C. elegans. Interestingly, only the glutamate receptor-1 (GLR-1) subunit was expressed in all four interneurons in the tap withdrawal circuit. These studies demonstrate that the chemical synapses in the tap withdrawal circuit are glutamatergic and that a mutation in a glutamate receptor subunit produced significant defects in synapse formation resulting in the expression of abnormal behaviors in C. elegans.

Numerous studies have demonstrated that an increase or decrease in the number of AMPA-type glutamate receptors at excitatory synapses contributes to several forms of synaptic plasticity, including learning and memory formation. Several studies have demonstrated a number of cellular mechanisms are involved in regulating the density of glutamate receptors at
the vertebrate synapse. Increases in the number of glutamate receptor-1 (GluR1) containing AMPA type glutamate receptors at vertebrate synapses has been shown to be regulated by the phosphorylation of the carboxy-terminus of the GluR1 subunit by a calmodulin dependent kinase II (CaMKII; Malinow, Shulman and Tsien, 1989), whereas decreases in GluR1 containing AMPA receptors at synapses have been shown to be regulated by endocytosis of clathrin coated vesicles (Carroll et al., 1999b), ubiquitination of GluR1 subunits (Burbea et al., 2002) and dephosphorylation of carboxy-terminus of the GluR1 subunit (Lee, Kameyama, Huganir and Bear, 1998). The density of GLR-1 containing AMPA type glutamate receptors at synapses in *C. elegans* are regulated by a number of similar molecular factors. These factors include the GLR-1 trafficking protein CaMKII (*unc-43*; Rongo and Kaplan, 1999), the PDZ (postsynaptic density-95, discs-large, ZO-1 homologous) domain GLR-1 trafficking protein LIN-10 (Rongo et al., 1998), the soluble clathrin adaptor protein AP180 (*unc-11*; Burbea et al., 2002), ubiquitin and the E3 ligase APC (Juo and Kaplan, 2004). Thus, the number of AMPA type glutamate receptors at vertebrate and invertebrate synapses, are dynamically regulated through similar molecular mechanisms.

Changes in environmental experiences have been shown to produce significant changes at excitatory synapses in the vertebrate central nervous system. The best studied forms of activity dependent plasticity in vertebrates are long-term potentiation (LTP) and long-term depression (LTD). These forms of activity dependent plasticity are associated with changes in the distribution of postsynaptic AMPA type glutamate receptors and subserve a number of functions during development such as experience dependent plasticity and learning and memory formation (Malenka and Bear, 2004). Previous studies have shown LTP is associated with an increase in AMPA receptor insertion at synapses, whereas LTD is associated with a decrease in AMPA receptors at synapses (Luscher et al., 1999; Carroll, Lissin, von Zastrow...
and Nicoll, 1999a). An increase in GluR1 subunit containing AMPA type glutamate receptors in hippocampal cells has been shown to mediate the synaptic strengthening observed during LTP (Luscher and Frerking, 2001). It has been hypothesized that this increase in GluR1 subunit containing AMPA receptors at synapses is achieved by an increase in the phosphorylation of GluR1 subunits, perhaps by CaMKII (Derkach, Barria and Soderling, 1999; Soderling, 2000). The decrease in AMPA type glutamate receptors observed with LTD has been shown to be achieved by dynamin-dependent endocytosis of clathrin coated vesicles (Carroll et al., 1999b), ubiquitination and degradation of AMPA receptors (Burbea et al, 2002) and a decrease in the phosphorylation of GluR1 subunits at the postsynaptic plasma membrane (Lee, Kameyama, Huganir and Bear, 1998). These forms of activity dependent plasticity demonstrate the dynamic nature of the vertebrate postsynaptic membrane and the ability of it to modify its molecular components as a result of environmental experience.

Previous studies have shown the presence of activity dependent plasticity within the excitatory synapses of tap withdrawal circuit in C. elegans as a result of changes in environmental experience during larval development. Worms raised in a mechanosensory deprived environment (i.e. 1 worm per Petrie plate) are significantly smaller in size, slower to lay eggs and had smaller initial responses to the tap stimulus compared to worms raised in colonies (i.e. 20 worms per Petrie plate; Rose et al., 2005). The decreased initial response to the tap stimulus and the smaller worm size observed in wild-type C. elegans raised in isolation was not observed in isolated raised worms carrying a mutation in glr-1 [glr-1(n2461)], suggesting a developmental role for GLR-1. In addition, worms carrying chimeric receptors made up of GLR-1 tagged to green fluorescent protein (GLR-1::GFP) expressed significantly less GLR-1::GFP when raised in isolation compared to colony raised worms.(Rose et al., 2005). Presynaptic changes were also quantified using a GFP tagged synaptobrevin-1
(presynaptic vesicular membrane transport protein; SNB-1::GFP) whose expression was restricted to the touch cells in the tap withdrawal circuit by using a mechanosensory (Mec) promoter. Worms raised in isolation expressed significantly less SNB-1::GFP compared to worms raised in colonies (Rose et al., 2005). These results suggest the importance of mechanosensory stimulation during *C. elegans* development for normal synaptic formation in the tap withdrawal circuit. Interestingly, the delivery of brief mechanical stimulation to isolate raised worms during larval development rescued both the behavioral deficit and decreased expression of GLR-1::GFP; however the decreased expression of SNB-1::GFP was not rescued by the delivery brief mechanical stimulation during larval development. Thus, similar to mammalian model systems, *C. elegans* exhibit experience dependent plasticity during development.

Adult activity dependent plasticity within the excitatory synapses of the tap withdrawal circuit of *C. elegans* was also observed by changes in environmental experiences. Rose et al. (2003) showed that the formation of long-term memory in *C. elegans* was dependent on the expression of GLR-1. Worms carrying a mutation in *glr-1* or a lack of glutamate activity by administering DNQX (6,7-Dinitroquinoxaline-2,3-dione; a non-NMDA glutamate receptor antagonist) to wild type worms during spaced habituation training, resulted in the abolishment of 24 hour long-term memory to the tap stimulus (Rose, Kaun Chen and Rankin, 2003). In addition, worms that were given long-term memory training expressed significantly less GLR-1::GFP 24 hours after spaced habituation training compared to untrained control worms (Rose et al., 2003). No difference in the expression of SNB-1::GFP was observed in the sensory neurons of worms that had received spaced habituation training 24 hours prior to testing compared to untrained control worms. These results demonstrate that long-term memory in *C. elegans* is dependent on the expression of *glr-1* and that spaced habituation training produces a
down regulation of glutamate receptors in the interneurons of adult *C. elegans*. Thus, similar to larval worms, adult worms also exhibit activity dependent plasticity at the excitatory synapses in the tap withdrawal circuit.

**OVERVIEW OF OBJECTIVES**

The significant behavioral and molecular effects observed in *C. elegans* raised in sensory deprived environments, and the fact that these effects were rescued by the delivery of brief mechanical stimulation, suggested the importance for early mechanosensory experience for normal behavioral and molecular development in *C. elegans*. This led us to consider how an enhancement in mechanosensory stimulation might affect the behavioral and molecular development in *C. elegans*. I hypothesized that an enhancement in mechanosensory experience during larval development would produce significant changes in the tap withdrawal behavioral response and GLR-1 distribution in adult *C. elegans*. To investigate this hypothesis, I performed a series of five experiments.

**Experiment 1: Early Larval Mechanosensory Stimulation had Different Effects on Adult Behavior at Three Adult Stages.** Pilot work suggested that early spaced stimulation [5 blocks of 20 taps delivered at a 60 second inter-stimulus interval] delivered during larval stage 1 (L1) produced a significant enhancement in the tap withdrawal response when worms were tested at 3 days, no behavioral difference at 4 days, and a significant depressed behavioral response at 5 days of age (Kaun and Rankin pers. comm.). For this experiment I modified the protocol by testing one third of the worms that had received spaced stimulation during L1 at 3 days, one third at 4 days, and one third at 5 days of age. Tap withdrawal responses were compared to an age matched control group that had received a single tap at the end of the 5th stimulation block.
The results from this experiment showed that spaced stimulation delivered during L1 produced an enhanced tap withdrawal response at 3 days, no behavioral difference at 4 days and a depressed tap withdrawal response at 5 days of age.

**Experiment 2: To Determine the Role of Glutamate in the Effects of Early Stimulation on Adult Behavior.** Previous work established the necessity for glutamate activity in *C. elegans* during development and in the formation of 24 hour long-term memory (Rose et al., 2003; Rose et al., 2005). In this experiment, I investigated whether glutamate transmission was necessary for the adult behaviors observed in L1 stimulated worms. This was accomplished by delivering spaced stimulation during L1 to worms carrying a nonsense mutation in *glr-1* or wild-type N2 worms stimulated in the presence of the glutamate antagonist DNQX. In addition, I investigated whether the expression of the post synaptic GLR-1 and presynaptic SNB-1 were affected as a result of L1 spaced stimulation by quantifying the total area of GLR-1::GFP and SNB-1 GFP. Furthermore, I measured whether spaced stimulation altered the levels of *glr-1* mRNA. From this series of experiments I was able to demonstrate that glutamate plays a critical role during development and showed the effects early spaced mechanosensory stimulation had on the expression of the GLR-1 protein and *glr-1* mRNA levels.

**Experiment 3: To Determine the Presence of a Critical Period for the 3 Day Enhanced and 5 Day Depressed Tap Withdrawal Behaviors.** Pilot work done showed that the enhanced tap withdrawal response observed in 3 day old worms that had received spaced stimulation during L1 was not observed when spaced stimulation was delivered during larval stage 3 (L3). This led us to hypothesize the presence of a critical period for the enhanced 3 day
old behavioral response early in *C. elegans* larval development. To investigate this, the same spaced stimulation paradigm in experiment one was performed during L2, L3 or L4 and the tap withdrawal behavior and total area of GLR-1::GFP was measured at 3 days, 4 days and 5 days of age and compared to an age matched control group. The results from this series of experiments showed the presence of a critical period during L1 for the 3 day enhanced tap withdrawal behavior and GLR-1::GFP distribution. The 5 day depressed behavioral response and GLR-1::GFP distribution was observed when stimulation was delivered during L1, L2 and L4, suggesting no critical period for this effect. No difference in the tap withdrawal behavior and GLR-1::GFP distribution was observed in 4 day old worms when stimulation was delivered during any of the four larval stages.

**Experiment 4: To Determine the Sensitivity of the 3 Day Enhanced Behavioral Response and 5 Day Depressed Behavioral Response to Reconsolidation Blockade.** When a memory is retrieved it returns to a labile state and is vulnerable to manipulations that interfere with memory reconsolidation (i.e. inhibition of protein synthesis; Nader, Schafe and Le Doux, 2000). Recently Rose and Rankin (2005) demonstrated that the formation of long-term memory to the tap stimulus in *C. elegans* is sensitive to disruption by inhibiting protein synthesis following memory reactivation. In this experiment, I investigated whether the significant changes observed in adult worms stimulated during larval development were sensitive to manipulations interfering with the reconsolidation of memories. To accomplish this spaced stimulation was delivered during L1. 10 reminder taps were delivered 24 hours later during L3. Heat shock was administered immediately after the 10 reminder tap stimuli, and the tap withdrawal behavior of 3 day and 5 day old worms were tested and compared to an age matched control group which did not receive heat shock during L3. In an addition, I
examined whether an increase in the time between the delivery of spaced stimulation and the reminder taps affected the sensitivity of 5 day old worms to reconsolidation blockade. This was done by delivering spaced stimulation during L1. 10 reminder taps were delivered 72 hours later at 4 days of age followed by heat shock. The tap withdrawal behavior of 5 day old worms was tested and compared to an age matched control group which did not receive heat shock at 4 days. This experiment demonstrated that the 5 day depressed behavioral response was sensitive to reconsolidation blockade; however the 3 day enhanced behavioral response was insensitive to reconsolidation blockade.

Experiment 5: Different Patterns of Stimulation Delivered During L1 Affected the 3 Day Enhanced and 5 Day Depressed Tap Withdrawal Behaviors. The formation of long-term memory to the tap stimulus has been shown to be dependent on the delivery of spaced stimulation 24 hours prior to testing (Beck and Rankin, 1997; Rose et al., 2002). When massed stimulation was delivered in lieu of spaced, C. elegans did not form long-term memory to the tap stimulus. In this experiment, I proposed to investigate whether a change in the temporal patterning of stimuli delivered during L1 affected the tap withdrawal response of adult worms. To accomplish this, one of four types of stimulation paradigms were delivered during L1: (1) massed stimulation at a 60 second inter-stimulus interval; (2) massed stimulation at a 204 second inter-stimulus interval; (3) massed stimulation at a 10 second inter-stimulus interval; (4) spaced stimulation at a 10 second inter-stimulus interval. The tap withdrawal response for stimulated adult worms was measured at 3 days, 4 days and 5 days of age and compared to an age matched control group. From this experiment I showed that the 5 day depressed and 3 day enhanced behavioral response were dependent on the delivery of spaced stimulation at a long inter-stimulus interval (i.e. 60 seconds); however the delivery of spaced stimulation at a short
inter-stimulus (i.e. 10 seconds) interval produced a significant depression in 3 day old worms that was found to be insensitive to reconsolidation blockade.
METHODS

SUBJECTS

A total of 1,838 *C. elegans* Bristol (N2; *Caenorhabditis elegans* Genetic Center, University of Minnesota, Minneapolis, MN) were used in these experiments. To investigate the role of glutamate in development we used a *C. elegans* strain carrying a nonsense mutation in glutamate receptor-1 (*glr-1*(*n2461*); a homologue of a kainite/AMP A-type glutamate receptor expressed on the interneurons of the tap withdrawal circuit; *n* = 120; *Caenorhabditis elegans* Genetic Center). The *n2461* mutation is located on chromosome III and corresponds to a nonsense mutation in codon 807 resulting in a premature stop. The truncated GLR-1 protein lacks the flip/flop domain, the last transmembrane and the cytoplasmic tail (Hart et al., 1995). The identical phenotypes observed in *glr-1* homozygotes and the *glr-1*/*nDf20* heterozygotes, suggests that the *n2461* mutation causes severe or complete loss of gene function (Hart et al., 1995). To quantify changes at the synapse, two transgenic *C. elegans* strains were used: GLR-1::GFP (J. Kaplan, Harvard University, Boston MA) and pMec-7::SNB-1::GFP (M. Nonet). Postsynaptic changes were visualized using GLR-1::GFP (*n* = 285) worms, which contain chimeric receptors made up of GLR-1 tagged with GFP. The *gfp* coding sequences were inserted in frame, 16 codons from the cytoplasmic tail of *glr-1* and the construct was integrated into the X chromosome of *C. elegans*. GLR-1::GFP was expressed in the interneurons under the control of the *glr-1* promoter. Chimeric GLR-1::GFP receptors retain glutamate receptor function *in vivo*, as they have been shown to rescue the nose touch deficiency observed in *glr-1*(*n2461*) mutants (Rongo et al., 1998). Presynaptic changes were visualized using a GFP tagged synaptobrevin-1 (*snb-1*; M. Nonet, Washington University, St. Louis, MI). SNB-1 is a protein associated with synaptic vesicles that plays a role in regulating
vesicle fusion at the synaptic terminal. pMec-7:: SNB-1::GFP (n = 67) was expressed under the control of the mec-7 promoter (pMec-7::SNB-1::GFP) which targets GFP expression to the six mechanosensory neurons within the tap withdrawal circuit.

REARING CONDITIONS

Behavior Assays: Age synchronized C. elegans strains were made by placing approximately 20 adult worms on two 55 mm Petrie plates filled with 10 mL Nematode Growth Medium (NGM) streaked with Escherichia coli (E. coli) OP50 as described by Brenner (1974). Adults were removed from the plate once approximately 40 eggs were counted on the plate (elapsed time 30 – 45 minutes).

Imaging Assays: Age synchronized C. elegans strains were made by placing approximately 10 adult worms on six 55 mm Petrie plates filled with NGM and streaked with E. coli OP50. Adults were removed from the plate once approximately 10 eggs were counted on the plate (elapsed time 20 – 30 minutes).

Real-time PCR Assays: Age synchronized colonies were made by placing approximately 30 adult worms on 16 90 mm plates filled with 30 mL NGM and streaked with E. coli OP50. Since worms were not transferred to individual plates during L3, larger plates were used to minimize the mechanosensory stimulation delivered from contact with other worms on the plate. Adults were removed once approximately 100 eggs were counted on each plate (elapsed time 60 – 90 minutes).
For all three experimental assays, the plates were divided into two groups: stimulated and unstimulated (i.e. control). For imaging and RT-PCR, each of the two groups (i.e. stimulated and unstimulated) were further divided into three groups corresponding to the three adult stages tested. The colonies were maintained in an incubator at a constant temperature (20 degrees Centigrade) and constant humidity (approximately 35 – 40%), until the worms were at the larval stage for delivery of spaced stimulation.

BEHAVIORAL STIMULATION AND TESTING APPARATUS

Figure 3. Apparatus to Produce the Tap Stimulus. Worms swim on agar-filled Petrie 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 Petrie 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.
Larval stimulation for behavior and imaging assays and adult behavior testing was performed on worms using a stereomicroscope (Wild Leitz, Canada, Ltd., Model M3Z). All testing responses were recorded by a digital camera (Panasonic Digital 5100) connected to a VCR (Panasonic AG 1960) and a monitor (NEC). In all recorded conditions a time-date generator (Panasonic WJ-810) superimposed the time and date on the video record as well as a digital clock to ensure precise stimulus delivery. Petrie plates containing individual subjects were placed in a tapping apparatus on top of the microscope stage supported by a plastic handle and connected to a micromanipulator (Marhouser Model MM33), which was secured to the table by a magnet to allow for smooth consistent movement of the plate in order to kept the subject in the video camera’s field of view throughout testing. A mechanical tapper produced vibrational stimuli with an electromagnet relay triggered by a Grass S88 stimulus generator (Grass Instruments, Quincy, MA) controlled by the experimenter. The wire arm was positioned in such a way that a mechanical tap stimulus was delivered halfway up the side of the Petrie dish containing the worms. Each mechanical tap exerted approximately 1 – 2 Newton’s of force onto the plate.

LARVAL STIMULATION AND ADULT TESTING

Stimulation was delivered during one of the four larval stages. See Table 1 for colony stimulation and transfer times. For both the behavior and imaging experiments, Petrie plates containing a colony of larval worms were placed on the microscope stage and a mechanical tap stimulus was delivered to the side of a Petrie plate. For RT- PCR experiments, mechanosensory stimulation was delivered simultaneously to Petrie plates containing a colony
of larval worms by placing the Petrie plates in a Tupperware box and dropping the box onto a table from a height of 50 mm.

<table>
<thead>
<tr>
<th>Larval Stage at Spaced Stimulation Training</th>
<th>Time at Start of Spaced Stimulation Training (hours after egg laying)</th>
<th>Larval Stage at Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>11.5 - 13</td>
<td>L3</td>
</tr>
<tr>
<td>L2</td>
<td>25 - 27</td>
<td>L3</td>
</tr>
<tr>
<td>L3</td>
<td>36 - 38</td>
<td>L3</td>
</tr>
<tr>
<td>L4</td>
<td>45 - 48</td>
<td>L4</td>
</tr>
</tbody>
</table>

Table 1. Developmental Training Transfer Times. This table outlines the time in hours after egg-lay when delivery of spaced stimulation was initiated, and the larval stage worms were transferred during for experimental assays. For real-time PCR assays, worms were not transferred to individual plates.

For behavior, imaging and RT-PCR assays, experimental worms were stimulated using either a spaced stimulation protocol which consisted of 5 blocks of 20 mechanical tap stimuli delivered at a 60 second inter-stimulus interval. Each block was followed by a one hour rest period. Massed stimulation consisted of 100 mechanical tap stimuli delivered at a 60 second inter-stimulus interval (Figure 4). The unstimulated control worms received a single tap stimulus at the end of the last stimulation block. After training, stimulated and control colony plates were placed on a padded box on a shelf to minimize vibrational stimuli.

For behavior and imaging assays, both stimulated and control worms were transferred to individual plates during L3 to individual Petrie plates filled with 10 mL of NGM and streaked with OP50 (with the exception of L4 stimulated worms which were transferred during L4). Behavior testing consisted of 10 taps delivered at a 60 second inter-stimulus interval using the same behavioral stimulation apparatus and was performed at three adult stages: 3
days old (60 - 64 hours after egg lay), 4 days old (84 – 88 hours after egg lay) and 5 days old (108 – 112 hours after egg lay). To ensure the accuracy of the data obtained from the behavioral and imaging experiments, each experimental assay was replicated a minimum of three times.

**Massed Stimulation**

<table>
<thead>
<tr>
<th>Test 1/3</th>
<th>3 Day Old</th>
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</thead>
<tbody>
<tr>
<td>Transfer in L3 (24 hr rest)</td>
<td></td>
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<tr>
<td>Single tap to control plate</td>
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<table>
<thead>
<tr>
<th>Test 1/3</th>
<th>4 Day Old</th>
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<tbody>
<tr>
<td>Transfer in L3 (24 hr rest)</td>
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<tr>
<td>Single tap to control plate</td>
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<tr>
<th>Test 1/3</th>
<th>5 Day Old</th>
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<tbody>
<tr>
<td>Transfer in L3 (24 hr rest)</td>
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<tr>
<td>Single tap to control plate</td>
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</table>

**Spaced Stimulation**

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<tbody>
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<td></td>
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<tr>
<td>Single tap to control plate</td>
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</table>

<table>
<thead>
<tr>
<th>Test 1/3</th>
<th>4 Day Old</th>
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<tr>
<td>Transfer in L3 (24 hr rest)</td>
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<td>Single tap to control plate</td>
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<tr>
<th>Test 1/3</th>
<th>5 Day Old</th>
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<tbody>
<tr>
<td>Transfer in L3 (24 hr rest)</td>
<td></td>
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<tr>
<td>Single tap to control plate</td>
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**Figure 4. Massed and Pattern Stimulation Paradigms.** Top illustration shows massed stimulation protocol, while bottom illustration shows the spaced stimulation protocol. Stimulation was delivered during one of the four larval stages. Worms were transferred to individual plates 24 hours after testing for L1 and L2 stimulated worms and 1 hour after L3 or L4 stimulation was delivered. Testing consisted of 10 taps delivered at a 60s ISI to both stimulated and control worms. Response magnitudes during testing were compared between groups.

For studies on the role of DNQX on the tap withdrawal behavior of L1 stimulated 3 day, 4 day and 5 day old worms, the drug was dissolved in 35 mM NaOH to prepare a 10 mM DNQX solution. Previous experiments showed no differences in the reversal behavior of worms treated with different dilutions of DNQX (see Figure 23 in Appendix 1 for DNQX and NaOH dose response curves) therefore a high drug concentration was used to ensure drug
penetration of the *C. elegans* cuticle. Approximately one hour prior to L1 stimulation (10.5 hours post egg lay) 500ul of either 10 mM DNQX or 35 mM NaOH (vehicle) was placed onto the surface of agar plates containing *C. elegans* embryos and *E. coli* thus allowing enough time for drug containing liquid medium to dry on the surface of the agar before the larval worms hatched from the eggs. For the drug groups, the 500ul of 10mM DNQX applied to 10 mL of agar made the effective external concentration in the worm’s environment 476 μM (Chen, pers. comm.). One hour following stimulation (18.5 hours after egg lay), worms were transferred to a new colony plate streaked with *E. coli*. Worms were transferred to individual plates during L3 and adult testing was performed as previously described.

**SCORING AND ANALYSIS**

The reversal response magnitudes (i.e. worm swam backwards in response to the tap stimulus within one second after the tap stimulus was delivered) during testing of stimulated and control worms were videotaped and scored using stop-frame video analysis and scored onto acetate sheets. In addition, for each worm videotaped a worm length tracing was made by measuring the worm from the tip of the head to the tip of the tail. The acetate sheets containing the tracings of reversal response magnitudes were scanned (UMAX Astra 2100U) into a Macintosh computer using either DeskScan II or VistaImage software. Tracings were measured with the public-domain NIH image program and all data analyses were performed in Statview 4.5. Reversal response magnitudes values were averaged across the test phase and standardized to wormlength. Where more than two groups were compared, ANOVA’s were used. The responses of experimental and control groups were compared using a Fisher’s PLSD Test.
In this research it is difficult to compare absolute values across different experiments due to the variability in environmental conditions that can occur on different days. Previous work in our lab has shown that fluctuations in humidity and temperature can produce significant changes in behavior. In addition, these assays are sensitive to extraneous vibrational stimuli. Work in our lab has shown that the exposure of worms to any pattern of vibration after stimulation delivery abolishes the behavioral effect being investigated. In these experiments, great lengths were taken to minimize these effects; all experiments were conducted in a temperature and humidity controlled environment, control and experimental worms were run on the same day under the same environmental conditions, vibrationally insulated boxes were used to maintain worm colonies and after stimulation was delivered worm colonies were maintained on a vibrationally insulated hanging shelf.

CONFOCAL MICROSCOPY AND GFP QUANTIFICATION

Membrane and non membrane bound GLR-1::GFP and pMec-7::SNB-1::GFP were quantified at the University of British Columbia BioImaging Facility. For imaging GFP transgenic worms, strains were immobilized in Lab-Tek II 4 chamber dishes (Fisher) using 12μL of 2,3-butanedione monoxime. Images were taken using the Bio-Rad Radiance Plus on an inverted Zeiss-Axiovert with DIC Optics (Bio-Rad) equipped with a Krypton/Argon laser. 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 512 X 512 pixel field of view with optical sections collected at 0.4μm intervals using a 60x oil lens.

The GLR-1::GFP strain expressed GFP along the ventral nerve cord and images were collected along the posterior portion of the ventral nerve cord from the tail to the vulva.

Images of GLR-1::GFP distributions were composed of 10 - 40 optical sections for each
segment, depending on the age of the worm. Capturing of adult GLR-1::GFP images was consistently acquired with the following microscope settings: gain = 24%; iris = 1.7; laser = 32%. Less GLR-1::GFP is expressed in L3 staged worms compared to adult worms due to their smaller size. Therefore a single image stack comprised of 10 – 15 optical sections was collected with the following microscope settings: gain = 24%; iris = 2.2; laser = 44% due to the lesser amount of GLR-1::GFP as a result of the decrease in worm size. The pMec-7::SNB-1::GFP strain expressed GFP in the touch cells and a single image stack comprised of 10 – 20 optical sections was collected from the region just posterior to the vulva. Due to the faint expression of pMec-7::SNB-1::GFP, images were captured with a gain of 24%, iris of 2.2 and a laser of 44%. Each stack of images was then compiled into a single projection image which was then used for further analyses.

A researcher blind to the treatment groups measured the GFP expression in the images using NIH Image 1.61. In Image J a threshold adjustment produced high-contrast images in black and white to allow for viewing of faint GFP. Threshold adjustments were individually optimized for each image stack, and were therefore not the same for all image stacks. Area measurements for each region of GFP expression were calculated by outlining the GFP expressing region and using the area measure function in Image J. Measurements were entered into Statview 4.5 for statistical analyses.

REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from 3 day, 4 day or 5 day old wild-type stimulated or control worms by homogenization in Trizol (Invitrogen). Single-stranded cDNA was generated from 1μg of RNA using SuperScript II Reverse Transcriptase (Invitrogen).
PCR: Normal PCR amplification was done on first-strand cDNA in a 10 μl reaction volume containing Taq DNA polymerase (Invitrogen) and consisted of 35 cycles of a denaturing step at 94 °C for 3 min; a two-step sequence of 94 °C for 30 seconds followed immediately by a step at 58 °C for 30 seconds and a 1 minute step at 72 °C. Forward (GGA GAG GTT CTG GTT TTG ATT GA) and reverse (TCG AGT ACG AAG ATF TCT CCA AAG) primers were selected for the C. elegans glr-1 gene (Invitrogen Life Technologies). The relative levels of glr-1 mRNA for stimulated and control worms were normalized to the levels of act-1 actin mRNA. Forward (TTG CCC CAT CAA CCA TGA A) and reverse (CTC CGA TCC AGA CGG AGT ACT T) primers were selected for the C. elegans act-1 gene (Invitrogen Life Technologies). After a final extension at 72 °C for 10 minutes, the PCR products were resolved on a 1.5% agarose gel containing ethidium bromide.

Real-Time PCR: Real-time PCR was performed using the same primers as mentioned earlier. A 25 μl Master Mix containing 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 0.75 μl forward primer, 0.75 μl reverse primer and 9.75 μl of water was prepared to perform real-time PCR. 2 μl of cDNA at a concentration of either 100%, 50% or 25% was added to the PCR Master Mix to a final volume of 25μl. The following PCR protocol was used on the ABI Prism 7000 apparatus (Applied Biosystems): initial denaturation (10 minutes at 95°C), followed by a two step amplification program (15 seconds at 95°C, followed by 1 minute at 60°C) repeated 40 times. The number of transcripts for each gene was determined using gene-specific standard curves using ABI integrated software. act-1 was chosen as the reference gene. A ratio of the mRNA levels of control glr-1/act-1 was normalized to the ratio of mRNA levels of stimulated glr-1/act-1 (see Table 2 caption for calculation description of
calculations used to determine mRNA levels) Each real-time PCR run included a sample containing no cDNA and replicates of stimulated and control samples.
RESULTS

EXPERIMENT 1

*Early larval mechanosensory experience had different effects on adult behavior at three adult stages.*

Numerous studies have demonstrated changes in the behavior of vertebrates and invertebrates reared in sensory enhanced environment; however, whether these changes prolonged into adulthood, or whether there was a cost to the precocial development is unknown. In our initial series of experiments I investigated whether early mechanical stimulation delivered during larval development changed the behavioral response of adult *C. elegans* to the same mechanical stimulus. This was accomplished by delivering 5 blocks of spaced mechanical stimulation at a 60 second inter-stimulus interval over 340 minutes during L1 (12 hours post egg lay). This pattern of stimulation was used as it has been shown to produce long-term memory in adult worms (Rose et al., 2002). To investigate whether behavioral changes as a result of early larval experience prolonged into adulthood, the magnitude of the behavioral response to the tap stimulus was quantified at three adult stages (3 days, 4 days and 5 days of age) and compared to the magnitude of the behavioral response of an age matched unstimulated control group which had received a single tap stimulus at the end of the spaced stimulation protocol. The results from three independent experiments showed that the delivery of early mechanical spaced stimulation during L1 significantly altered the tap withdrawal behavior of 3 day and 5 day old adult worms (Figure 5). In addition, the behavioral response observed for both stimulated and control worms, was not consistent across all three ages tested. An overall ANOVA comparing the magnitude of the reversal response to tap by the total worm
length between stimulated and control worms showed that worms that had received mechanical stimulation during L1 exhibited a significant enhancement in the behavioral response at 3 days (n = 26 for stimulated and control; p ≤ 0.0329), no difference in the tap withdrawal response magnitude at 4 days (n = 25 for stimulated; n = 24 for control; p ≤ 0.8141), and a significant depression in the behavioral response at 5 days of age (n = 18 for stimulated and control; p ≤ 0.0407). Thus, an enhancement in mechanosensory experience produced significant changes in wild-type C. elegans adult behavior that was not consistent across the three ages tested.
Figure 5. Effects of Spaced Stimulation during L1 on Adult C. elegans Behavior

Line graphs illustrate the average reversal response magnitudes for each of the 10 test taps for A) 3 day old worms stimulated during L1 and its age matched single tap control group, B) 4 day old worms stimulated during L1 and its age matched single tap control group, and C) 5 day old worms stimulated during L1 and its age matched single tap control group. Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms. (D). * = p \leq 0.05.

The significant changes in the tap withdrawal response behavior of 3 day and 5 day old C. elegans' stimulated during L1 may be due to a change in the normal developmental pattern. To explore this possibility, I performed a within condition comparative analysis between 3 day
and 5 day old worms. A Fisher’s planned comparisons showed a significant increase in the magnitude of the tap withdrawal response of control worms from 3 day to 5 days of age (p ≤ 0.0383) and a significant decrease in the magnitude of the tap withdrawal response of L1 stimulated worms from 3 day to 5 days of age (p ≤ 0.0372; Figure 6). The opposing behavioral trends observed between stimulated and control worms suggests a role for early experience in the functional development of the *C. elegans* nervous system.

**Figure 6. Early Stimulation Alters Wild-Type Adult Behavior.**
Mean response magnitudes over worm length to the 10 test taps were compared for control worms (1st bar group) at all three ages tested and for L1 stimulated worms (2nd bar group) at all three ages tested. * = p ≤ 0.05.
EXPERIMENT 2

To determine the role of glutamate in the effects of early mechanosensory stimulation on adult behavior.

The absence of sensory experience during larval development has been shown to produce profound consequences on the biology and function of the *C. elegans* nervous system. Worms raised in isolation exhibited smaller initial responses to the tap stimulus and a decrease in GLR-1::GFP and SNB-1::GFP distribution compared to worms raised in colonies (Rose et al., 2005). In this experiment, I investigated whether glutamate activity was required to produce the behavioral differences observed in 3 day and 5 day old worms that had received spaced stimulation during L1. I also explored whether an enhancement in mechanosensory experience during L1 resulted in changes in the distribution of GLR-1 and SNB-1.

To accomplish this, I began by investigating whether a strain carrying a mutation in *glr-1* [glr-1(n2461)] exhibited the same changes in behavior as L1 stimulated wild-type worms. *glr-1(n2461)* worms were given spaced stimulation during L1 and one third were tested at 3 days, one third at 4 days, and the final third at 5 days of age. The tap withdrawal response was compared to an age matched unstimulated control *glr-1(n2461)* mutant. An overall ANOVA comparing the magnitude of the reversal response to tap by the total worm length between stimulated and control worms for three independent runs showed that spaced stimulation delivered during L1 to worms carrying a mutation in *glr-1* worms produced no behavioral difference at 3 days (n = 20 for stimulated and control p ≤ 0.8842), 4 days (n = 19 for stimulated; n = 22 for control; p ≤ 0.7912), and 5 days of age (n = 19 for stimulated; n = 20 for control; p ≤ 0.8731). In addition, the magnitude of the adult responses to the 10 tap stimuli in the *glr-1* mutant strain were smaller compared to the responses of wild type worms (see Figure...
5). This is because the neural circuit for tap is composed of two competing circuits that are connected by both electrical and chemical synapses. The circuit is driven by electrical synapses; however, the strength of each of the competing inputs is mediated, at least in part by activation of the glutamate receptors. When glutamate neurotransmission is disrupted the balance of electrical input driving the competing responses is hypothesized to be altered leading to smaller responses. These results suggest the regulation of the behavioral response of wild-type adult worms by glr-1.

![Graph showing the behavioral response of C. elegans worms](image)

**Figure 7. Spaced Stimulation Delivered During L1 to Adult C. elegans carrying a mutation in glr-1.**

No significant difference in the magnitude of the behavioral response to a tap was observed between stimulated and control *C. elegans* at all three ages. Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old *glr-1* mutant worms.

To confirm that the absence of the 3 day enhanced behavior and 5 day depressed behavior in L1 stimulated *glr-1(n2416)* mutants resulted from an absence of glutamate activity, wild-type worms were treated with the competitive non-NMDA glutamate receptor antagonist
DNQX (6,7-Dinitroquinoxaline-2,3 (1H, 4H)-dione; Sigma). DNQX has been shown to block neurotransmission at the *Aplysia* sensory neuron to motor neurons synapses (Dale and Kandel, 1993; Chitwood, LI and Glanzman, 2001) and the formation of 24 hour long-term memory in *C. elegans* (Rose et al., 2003).

Spaced stimulation was delivered during L1 to wild-type (N2) worms in the presence of 35 mM NaOH (vehicle) or 10 mM DNQX dissolved in 35 mM NaOH. Treated worms were tested at 3 day, 4 day and 5 days of age and were compared to an age matched unstimulated control group that had undergone the same drug treatments. An overall ANOVA comparing the magnitude of the reversal response to tap by the total worm length between stimulated and control worms showed that spaced stimulation delivered in the presence of DNQX during L1 did not produce any difference in response magnitudes at 3 days (n = 22 for stimulated and control p ≤ 0.5613), 4 days (n = 13 for stimulated and control; p ≤ 0.4884), and 5 days of age (n = 14 for stimulated and control; p ≤ 0.2611; Figure 8A); however worms treated with 35mM NaOH did exhibit a trend towards a behavioral enhancement at 3 days (n = 19 for stimulated and control; p ≤ 0.0518), no difference at 4 days (n = 12 for stimulated, n = 13 for control; p ≤ 0.0679), and a significant behavioral depression at 5 days of age (n = 14 for stimulated and control; p ≤ 0.0415; Figure 8B). Thus, exposure to the vehicle during L1 spaced stimulation had no toxic effect, whereas exposure to vehicle plus DNQX during L1 spaced stimulation blocked the expression of the 3 day behavioral enhancement and 5 day behavioral depression.

The difference in the tap withdrawal behaviors in worms treated with DNQX during stimulation and *glr-1(n2461)* mutant worms results from differences in the experimental protocols. DNQX and NaOH worms were exposed to additional handling stimulation during L1 due to changes in the experimental protocol that were needed to accommodate drug
delivery. In addition, there were differences in the testing environments between DNQX treated and \textit{glr-1(n2461)} mutant worms. DNQX treated worms were tested under wild-type environmental conditions with functioning glutamate receptors, whereas the mutation in \textit{glr-1} results in a non functioning GLR-1 receptor during testing. Despite the differences in the experimental protocols, the results from DNQX treated worms and \textit{glr-1(n2461)} mutants demonstrate the role of glutamate in producing the enhanced 3 day and depressed 5 day tap withdrawal behaviors.

\begin{figure}
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\caption{\textit{C. elegans} Stimulated During L1 in the Presence of DNQX or NaOH (vehicle).}

A) Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms exposed to DNQX during L1 stimulation. B) Differences between average response magnitudes standardized over worm length across the 10 test taps between control and stimulated worms were calculated for 3 day, 4 day and 5 day old worms exposed to NaOH during L1 stimulation. * = p \leq 0.05

The absence of the behavioral enhancement at 3 days and depression at 5 days in both \textit{glr-1(n2461)} worms and wild-type worms exposed to DNQX, suggests the regulation of these behaviors by the GLR-1 receptor. This led us to investigate whether early mechanical stimulation alters the expression of the GLR-1 receptor. To visualize changes in GLR-1
receptor distribution, chimeric worms carrying a GFP tagged GLR-1 were given spaced stimulation during L1 (Rongo and Kaplan, 1999). GLR-1::GFP has been shown to be localized to synaptic clusters along the ventral nerve cord and in the nerve ring (White et al., 1986; Burbea et al., 2002). The expression of GLR-1::GFP was determined by quantifying the total area of GFP puncta observed along the posterior ventral nerve cord in confocal images of L3 staged, 3 day, 4 day, and 5 day old worms stimulated during L1, and comparing it to an age matched control group. Spaced stimulation delivered during L1 produced a significant increase in the total area of GLR-1::GFP at L3 (n = 11 for stimulated; n = 13 for control; p ≤ 0.0491; Figure 9C) and at 3 days of age (n = 11 for stimulated and control; p ≤ 0.0287; Figure 9B). No difference in the total area of GLR-1::GFP was observed at 4 days (n = 13 for stimulated and control; p ≤ 0.4405; Figure 9B). A significant depression in the total area of GLR-1::GFP was observed in 5 day old worms that received spaced stimulation during L1 (n = 10 for stimulated; n = 9 for control; p ≤ 0.003; Figure 9B). These results demonstrate a positive correlation between the tap withdrawal behaviors of L1 stimulated worms to the total area of GLR-1::GFP observed along the posterior ventral nerve cord.
Figure 9. Spaced Stimulation during L1 Altered the Total Area of GLR-1::GFP in *C. elegans*

A) Confocal images of GLR-1::GFP postsynaptic expression on interneuron processes in the posterior ventral nerve cord of a worm receiving spaced stimulation during L1 and a unstimulated control worm at 3 days, 4 days, and 5 days of age. B) Differences between total area of GLR-1::GFP expression over the number of worms imaged between control (white bars) and stimulated (black bars) worms for 3 day, 4 day and 5 day old GLR-1::GFP worms. C) Difference between total area of GLR-1::GFP expression standardized over total worm length in the image between L1 stimulated and control L3 worms. * = p ≤ 0.05; ** = p ≤ 0.01.
The differences in the total area of GLR-1::GFP observed between worms that received spaced stimulated during L1 and control worms could be due a number of possibilities. Early mechanical stimulation may affect GLR-1 localization by either causing a re-distribution of GLR-1::GFP along the ventral nerve cord and in the nerve ring, an increase in GLR-1 ubiquitination and degradation, or a change in \(glr-1\) gene transcription. I began to explore these possibilities by using quantitative Real-Time PCR to compare \(glr-1\) mRNA levels between adult worms that had received spaced stimulation during L1 to unstimulated age matched control worms. 3 day old stimulated worms exhibited a significant increase in \(glr-1\) mRNA (mean = 2.072, \(n = 2\)), no difference in the expression of \(glr-1\) mRNA levels was observed at 4 days (mean = 1.026, \(n = 3\)) and 5 day old worms exhibited a significant decrease in \(glr-1\) mRNA levels (mean = 0.477, \(n = 4\); Table 2). These results demonstrate a positive correlation between the distribution of GLR-1::GFP and \(glr-1\) mRNA levels (Figure 10).
Table 2. Numerical Values Calculated from Real-Time PCR Runs for 3 Day, 4 Day and 5 Day Old Worms Stimulated During L1.

The numerical values in each column represent the calculated levels of *glr-1* mRNA in each diluted sample. 100% samples were not diluted with DEPC water, 50% samples were diluted in 50% DEPC water, and 25% samples were diluted in 75% DEPC water. The numerical values were first calculated by normalizing the stimulated and control *glr-1* mRNA values to the stimulated and control reference gene *act-1* mRNA values. To obtain a single numerical value representing the level of *glr-1* mRNA expression in stimulated worms, the calculated value of the stimulated *glr-1* mRNA/act-1 mRNA was normalized to the calculated value of the control *glr-1* mRNA/act-1 mRNA. Due to human error, some of the dilution values reported from the ABI Prism RT-PCR machine were slightly askew from the other reported dilution values (i.e. threshold value reported was much greater than expected; italicized values in tables 2A and 2C). This dissimilarity in numerical values was most likely due to human error and were not included in the calculations only if a corresponding dissimilarity was observed in the actin control gene of the same dilution. A) The dilution values calculated for both real-time PCR runs for 3 day old L1 stimulated worms. The numerical value listed below the table (i.e. 2.072) represents the average *glr-1* mRNA level for both runs. B) The dilution values calculated for the three real-time PCR runs for 4 day old L1 stimulated worms. The numerical value listed below the table (i.e. 1.026) represents the average *glr-1* mRNA level for the three runs. C) The dilution values calculated for four real-time PCR runs for 5 day old L1 stimulated worms. The numerical value listed below the table (i.e. 0.477) represents the average *glr-1* mRNA level for the four runs.
Figure 10. Spaced Stimulation during L1 Altered glr-1 mRNA Levels in C. elegans
Expression of glr-1 mRNA levels was determined using quantitative Real-time PCR. Each bar represents the level of expression of glr-1 mRNA in L1 stimulated worms normalized to the expression of glr-1 mRNA in control worms. β-actin was used as a control. Values over 1.5 were considered to be a significant in glr-1 mRNA levels in stimulated worms (region above top dotted line), values from 1 – 1.5 were considered as no difference in expression between the two conditions (between the two dotted lines), values 0 – 1 were considered to be a decrease in glr-1 mRNA levels in the stimulated worms (region below bottom dotted line). * = p ≤ 0.05

In a final set of experiments, I tested whether early stimulation affected presynaptic terminals in the same way it affected receptor expression by using a transgenic strain containing the presynaptic GFP tagged synaptobrevin-1 marker. Using confocal imaging I investigated whether L1 spaced stimulation altered the total area of pMec-7::SNB-1::GFP in tap sensory neurons. The expression of pMec-7::SNB-1::GFP was determined by quantifying the total area of GFP puncta observed in a region just posterior to the vulva in confocal images of L1 stimulated 3 day, 4 day and 5 day old worms and comparing it to an age matched control group. My results showed no significant differences in the expression of pMec-7::SNB-1::GFP between stimulated and unstimulated worms at 3 days (n = 13 for stimulated and control stimulated: p ≤ 0.8429), 4 days (n = 9 for stimulated; n = 10 for control; p ≤ 0.5313) and 5 days of age (n = 12 for stimulated; n = 10 for control; p ≤ 0.4896 Figure 11). This was not
surprising as we have not seen changes in pMec-7::SNB-1::GFP expression in response to mechanosensory stimulation in any of our studies to date (Rose et al., 2003; Rose et al., 2005)

Figure 11. Spaced Stimulation during L1 did Not Affect pMec-7::SNB-1::GFP Expression
A) Confocal images of the vesicles in tap sensory neuron terminals visualized with a synaptobrevin GFP marker showing a worm that had received spaced stimulation during L1 and a unstimulated control worm at 3, 4, and 5 days of age. B) Differences between total area of pMec-7::SNB-1::GFP expression over the number of worms imaged between control (white bars) and stimulated (black bars) worms for 3 day, 4 day and 5 day old pMec-7::SNB-1::GFP worms.
EXPERIMENT 3

To determine the presence of a critical period for the 3 day enhanced and 5 day depressed tap withdrawal behaviors

Critical Periods

Early experiences influence the development of the nervous system and the expression of adult behavior. It has been shown that organisms are susceptible or even need certain environmental experiences at particular phases during their lifetime in order for 'normal' development to occur. These phases are termed either a "critical period" to describe an abrupt decline in plasticity where the absence of the event results in abnormal development, or a "sensitive period" to describe a gradual decline or reduced plasticity where the organism is susceptible to certain experiences but will develop normally if the incident was not experienced (Newport, 2004).

The existence of critical periods have been demonstrated in humans for the visual, auditory and somatosensory systems (Newport, 2004). Studies investigating the effects of altering a sensory input (i.e. rearing in a restricted auditory or visual environment) showed that significant effects only occur when the sensory manipulation occurs during a critical period, whereas little or no effect is observed in mature animals exposed to similar sensory manipulations (Berardi, Pizzorusso and Maffei, 2000; Newport, 2004). Thus, only when environmental sensory experiences occur during a critical period is a profound effect on the nervous system and behavior observed.

Critical periods have been investigated using animal model systems. Rodents whose whiskers are clipped during the critical period when the development of the cortex region that receives input from the face results in a undeveloped cortex; however, if the whiskers are cut
off after the critical period or beyond, the whiskers grow back and the rodent still has touch sensation from the face. The exposure of young male passerine birds to the courtship songs of conspecific males during the critical period for song acquisition is necessary for them to be able to sing normally when they mature (Arnold, 1982). These studies demonstrate that exposure to appropriate stimuli during a critical period is necessary for the development of wild-type nervous system structures and behavior in adult vertebrates.

In this set of experiments, I investigated whether there was a critical period for the 3 day enhanced tap withdrawal response and total area of GLR-1::GFP and 5 day depressed tap withdrawal response and total area of GLR-1::GFP observed for worms that had received spaced stimulation during L1. This was accomplished by delivering spaced stimulation at a 60 second inter-stimulus interval over 340 minutes during either L2 (26 hours post egg-lay), L3 (36 hours post egg-lay) or L4 (45 hours post egg-lay), and testing the tap withdrawal response or quantifying the total area of GLR-1::GFP puncta at 3 days, 4 days and 5 days of age. The magnitude of the tap withdrawal response and the total area of GLR-1::GFP were compared to an age matched unstimulated control group.

**Spaced Stimulation Delivered During L2**

An overall ANOVA demonstrated that spaced stimulation delivered during L2 produced no difference between stimulated and control worms in the magnitude of the behavioral response to tap at 3 days (n = 20 for stimulated; n = 22 for control; p ≤ 0.2812) and 4 days (n = 19 for stimulated and control; p ≤ 0.9071; Figure 12A) or the total area of GLR-1::GFP at 3 days (n = 14 for stimulated; n = 12 for control; p ≤ 0.8858) and 4 days of age (n = 5 for stimulated; n = 4 for control; p ≤ 0.9532; Figure 12B). Five day old stimulated worms exhibited a significant depression in the behavioral response to tap (n = 18 for stimulated and
control, \( p \leq 0.0131 \) and in the total area of GLR-1::GFP compared to control worms (\( n = 10 \) for stimulated and control; \( p \leq 0.0416 \); Figure 12A and B).

### Figure 12. Effects of Spaced Stimulation during L2 on Behavior and GLR-1::GFP Expression

A) Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms that received spaced stimulation during L2. \( * = p \leq 0.05 \). B) Differences between total area of GLR-1::GFP expression over the number of worms imaged between L2 control and stimulated worms for 3 day, 4 day and 5 day old GLR-1::GFP transgenic worms. \( * = p \leq 0.05 \).

### Spaced Stimulation Delivered During L3

An overall ANOVA revealed that spaced stimulation delivered during L3 resulted in no significant difference in the magnitude of the behavioral response between stimulated and control worms at 3 days (\( n = 30 \) for stimulated and control; \( p \leq 0.8068 \)), 4 days (\( n = 22 \) for stimulated; \( n = 23 \) for control; \( p \leq 0.1393 \)) and 5 days (\( n = 42 \) for stimulated and control; \( p \leq 0.7852 \); Figure 13A). In addition, no difference in the total area of GLR-1::GFP was observed between stimulated and control worms for 3 days (\( n = 18 \) for stimulated; \( n = 13 \) for control; \( p \leq 0.5715 \)), 4 days (\( n = 11 \) for stimulated; \( n = 12 \) for control; \( p \leq 0.4906 \)) and 5 days of age (\( n = 11 \) for stimulated; \( n = 12 \) for control; \( p \leq 0.8414 \); Figure 13B).
Figure 13. Effects of Spaced Stimulation during L3 on Behavior and GLR-1::GFP Expression

A) Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms that received spaced stimulation during L3. * = p ≤ 0.05. B) Differences between total area of GLR-1::GFP expression over the number of worms imaged between L3 stimulated and control worms for 3 day, 4 day and 5 day old GLR-1::GFP transgenic worms.

The duration of a critical period during an organisms development can vary depending on the type of stimulus and the effects it produces (Berardi et al., 2000). The absence of the behavioral depression in 5 day old worms that received spaced stimulation during L3 led us to hypothesize the presence of a critical period early in L3 for the depressed behavioral effect. To test this hypothesis, I changed the spaced stimulation delivery start time from the beginning of L3 (i.e. 36 hours post egg lay) to the middle of L3 (39 hours post egg-lay). The tap withdrawal response was tested at 5 days of age and compared to an age matched control group. The results showed that changing the stimulation delivery start time produced no significant difference in the tap withdrawal response between 5 day stimulated and control worms (n = 10 for stimulated and control; p ≤ 0.7852; Figure 14). Thus, no 5 day behavioral depression was observed when spaced stimulation was delivered during L3.
Figure 14. Changing of the Time Stimulation Delivered During L3 Produced no Significant Behavioral Difference.
Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between control (white bar) and stimulated (black bar) 5 day old worms that received spaced stimulation starting in the middle of L3.

Spaced Stimulation Delivered During L4

An overall ANOVA showed that spaced stimulation delivered during L4 produced no significant difference in the magnitude of the tap withdrawal response between stimulated and control worms at 3 days (n = 21 for stimulated; n = 19 for control; p ≤ 0.6500) and 4 days (n = 18 for stimulated; n = 19 for control; p ≤ 0.7741; Figure 15A). In addition, no difference in the total area of GLR-1::GFP was observed between stimulated and control worms at 3 days (n = 12 for stimulated and control; p ≤ 0.9040) and 4 days of age (n = 9 for stimulated; n = 8 for control; p ≤ 0.7727; Figure 15B). Interestingly, a significant depression in the magnitude of the tap withdrawal response of L4 stimulated 5 day old worms (n = 36 for stimulated; n = 38 for control; p ≤ 0.0365; Figure 15A) and a significant decrease in the total area of GLR-1::GFP was observed in L4 stimulated 5 day old worms (n = 11 for stimulated; n = 10 for control; p ≤ 0.0444; Figure 15B). Thus, the delivery of spaced mechanical stimulation during L4, produced
a significant decrease in both the tap withdrawal behavior and total area of GLR-1::GFP in 5
day old worms.

Figure 15. **Effects of Spaced Stimulation during L4 on Behavior and GLR-1::GFP Expression**
A) Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms that received spaced stimulation during L4. * = p ≤ 0.05. B) Differences between total area of GLR-1::GFP expression over the number of worms imaged between L4 stimulated and control worms for 3 day, 4 day and 5 day old GLR-1::GFP transgenic worms. * = p ≤ 0.05.
EXPERIMENT 4

To determine the sensitivity of the 3 day enhanced and 5 day depressed behavioral response to reconsolidation blockade.

The results from experiment 3 demonstrate the presence of a critical period during L1 for the enhanced tap withdrawal response and total area of GLR-1::GFP observed in 3 day old worms that received spaced stimulation during L1. Interestingly, there appeared to be no critical period for the depressed tap withdrawal response observed in L1 stimulated 5 day old worms as the depression was observed when spaced stimulation was delivered during L1, L2 and L4. This depression is similar to what is seen as long-term memory when worms are trained using a very similar protocol at four days of age and tested at five days of age. This raises the question of whether the depression seen in L1, L2 and L4 represents long-term memory for the earlier training or whether it represents an effect of early experience on development. In adult worms long-term memory is dependent on the expression of glr-1. The data from figure 7 shows that the 5 day old depressed behavioral response in L1 stimulated worms is glr-1 dependent. One way to investigate whether the depression seen in five day old worms stimulated during L2 or L4 is memory is to test whether it is dependent on glutamate. To do this, L1 worms carrying a mutation in glr-1 [glr-1(n2461)] were stimulated during L2 or L4 and tested at 5 days of age. Our results showed that the significant depression seen in wild-type L2 and L4 stimulated worms was absent in the glr-(n2461)1 mutant C. elegans receiving stimulation during L2 (n = 10 for stimulated and control; p ≤ 0.5621; Figure 16B) and L4 (n = 10 for stimulated and control; p ≤ 0.5379; Figure 16D). These results demonstrate that similar to long-term memory the behavioral depression seen in L1, L2 and L4 stimulated worms was dependent on the expression of glr-1.
In adult worms, the memory that is produced by earlier training can be “erased” using a procedure called reconsolidation blockade (Rose and Rankin, 2005). Reconsolidation blockade involves the notion that when a memory is recalled it becomes labile again and can be eliminated by manipulations that interfere with memory consolidation (i.e. inhibition of protein synthesis or pharmacological blockade of transcription or translation). If the 5 day old depression seen in L1, L2 or L4 or the 3 day old enhancement seen in L1 stimulated worms represents memory then it should be sensitive to reconsolidation blockade. To test this worms were given spaced stimulation during L1 and then 24 hours later during L3 ten taps were delivered at a 60 second inter-stimulus interval, followed by 40 minutes of heat shock at 32 degrees centigrade. Heat shock was used to deliver a discrete inhibition of protein synthesis as it does not affect the ability of C. elegans to move or respond to tactile stimuli (Beck and Rankin, 1995). Worms were tested at 3 days and 5 days of age using 10 taps delivered at a 60 second inter-stimulus interval. If the enhancement at 3 days and depression at 5 days of age
following larval training is memory it should be “erased” by this procedure. The results showed the behavioral enhancement was observed in 3 day old worms that did not receive heat shock (n = 14 for stimulated; n = 15 for control; p ≤ 0.0274; Figure 17B) and for 3 day old worms that were given heat shock immediately following the reminder taps (n = 15 for stimulated; n = 13 for control; p ≤ 0.0282; Figure 17B). Thus, the behavioral enhancement at 3 days was insensitive to reconsolidation blockade. The behavioral depression was observed in 5 day old worms that did not receive heat shock (n = 17 for stimulated; n = 18 for control; p ≤ 0.0474; Figure 17D); however the administration of heat shock following reminder treatment disrupted the 5 day behavioral depression (n = 17 for stimulated and control; p ≤ 0.6703; Figure 17D). Therefore, the disruption of protein synthesis by administering heat shock after the 10 reminder taps prevented the expression of the depressed behavioral response to the tap stimulus at 5 days of age but not the expression of the enhanced behavioral response at 3 days of age.
Figure 17: The 3 Day Enhancement was Insensitive to Reconsolidation Blockade and the 5 Day Depression was Sensitive to Reconsolidation Blockade.
A) Reconsolidation protocol, stimulated worms received 5 blocks of 20 taps and 10 taps for reminder. Testing consisted of measuring the response of 3 day old worms to 10 taps; B) Average reversal response standardized to worm length for stimulated and control worms that received reminder training followed by heat shock or no shock; C) Reconsolidation protocol; stimulated worms received 5 blocks of 20 taps and 10 taps for reminder. Testing consisted of measuring the response of 5 day old worms to 10 taps; D) Average reversal response standardized to worm length for stimulated and control worms that received reminder training followed by heat shock or no shock. *=p<0.05.

In the previous experiment, the time between the 10 reminder taps and testing of 3 day old worms was 24 hours, whereas the time between the 10 reminder taps and testing of 5 day old worms was 72 hours. To test whether the sensitivity of 5 day old worms to reconsolidation blockade was due to the increased time between the reminder tap delivery and testing, worms were given spaced stimulation at a 60 second inter-stimulus interval during L1 followed by a 72 hour rest period. At 4 days of age 10 reminder taps were delivered at a 60 second inter-
stimulus interval followed by 40 minutes of heat shock at 32 degrees centigrade. Worms were then transferred to individual plates and tested 24 hours later at 5 days of age. Testing consisted of 10 taps delivered at a 60 second inter-stimulus interval. The results showed that the behavioral depression was observed in 5 day old worms that did not receive heat shock treatment (n = 14 for stimulated; n = 17 for control; p ≤ 0.0468; Figure 18B); however the administration of heat shock following reminder treatment disrupted the 5 day behavioral depression (n = 18 for stimulated and control; p ≤ 0.6756; Figure 18B). Thus, administration of heat shock abolished the significant behavioral depression in 5 day old L1 stimulated worms. Therefore, the increase in time between reminder tap delivery and testing did not affect the sensitivity of 5 day old worms to reconsolidation blockade. These results demonstrate that similar to the behavioral depression observed as a result of long-term habituation training, the depressed behavioral response seen in L1 stimulated 5 day old worms was sensitive to reconsolidation blockade; however the enhanced behavioral response of L1 stimulated 3 day old worms was insensitive to reconsolidation blockade.
Figure 18. Increasing the Time Between Delivery of Stimulation and Reminder Taps did not Change the Sensitivity of 5 Day Old Worms to Reconsolidation Blockade.
A) Reconsolidation protocol, stimulated worms received 5 blocks of 20 taps and 10 taps for reminder. Testing consisted of measuring the average response to 10 tap stimuli standardized over worm length; B) Average response during testing for control and stimulated worms that received reminder training flowed by heat shock or no shock.
EXPERIMENT 5

*Different Patterns of Stimulation Delivered During L1 Affected the 3 day enhanced and 5 day depressed Tap Withdrawal Behavior.*

Neurons encode sensory information temporally or spatially. In order to generate accurate representations of the world the brain must decode both types of sensory encoded information. To determine whether different patterns of mechanosensory stimulation delivered during L1 affected adult *C. elegans* behavior, I altered the temporal and spatial patterns of mechanosensory stimulation. Previous work from our lab has demonstrated that different patterns of stimulation activate different mechanisms (Broster and Rankin, 1994; Wicks and Rankin, 1996; Beck and Rankin, 1997). This series of experiments suggests that the same is true during development. Worms were stimulated during L1 using a form of mechanical stimulation that have previously been shown not to produce memory – massed stimulation at a 60 second inter-stimulus interval. The results showed that massed stimulation delivered during L1 at a 60 second inter-stimulus interval produced no behavioral difference between stimulated and control worms at 3 days (n = 20 for stimulated; n = 21 for control; p ≤ 0.9248), 4 days (n = 23 for stimulated; n = 22 for control; p ≤ 0.4416) and 5 days of age (n = 21 for stimulated and control; p ≤ 0.6765; Figure 19).
Figure 19. Effects of Massed Stimulation During L1 on Adult C. elegans Behavior
Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between stimulated (black bars) and control (white bars) worms was calculated for 3 day, 4 day and 5 day old worms that received massed stimulation at a 60 second inter-stimulus interval.

There are two differences between spaced and massed stimulation. First the temporal organization of spaced stimulation versus massed stimulation provides important information for neurons and different temporal patterns are encoded in different ways leading to different outcomes. The second difference is the duration of time of spaced stimulation versus massed stimulation. Spaced stimulation delivered at a 60 second inter-stimulus interval spans 340 minutes, whereas massed stimulation delivered at a 60 second inter-stimulus interval spans 100 minutes. In order to determine which of these factors was more important, I ran experiments altering the temporal patterns in 2 ways: (1) massed stimulation at a 204 second inter-stimulus interval; (2) massed stimulation at a 10 second inter-stimulus interval. The results showed that massed stimulation delivered at a 204 second inter-stimulus interval did not produce any difference in the tap withdrawal response between stimulated and control worms at 3 days (n =
39 for stimulated; \( n = 40 \) for control; \( p \leq 0.7532 \), 4 days (\( n = 27 \) for stimulated and control; \( p \leq 0.2504 \)) and 5 days of age (\( n = 35 \) for stimulated; \( n = 33 \) for control; \( p \leq 0.1866 \); Figure 20A).

In addition, no behavioral difference was observed between stimulated and control worms when massed stimulation was delivered at a 10 second inter-stimulus interval at 3 days (\( n = 15 \) for stimulated; \( n = 15 \) for control; \( p \leq 0.1578 \)), 4 days (\( n = 8 \) for stimulated; \( n = 7 \) for control; \( p \leq 0.8074 \)) and 5 days of age (\( n = 13 \) for stimulated; \( n = 12 \) for control; \( p \leq 0.8455 \); Figure 20B).

Thus, no behavioral differences were observed between stimulated and control worms for all three ages tested when stimulation was delivered in a single massed block during L1.

**Figure 20. Effects of 204 Second and 10 Second Massed Stimulation on Adult C. elegans Behavior**

A) Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between stimulated (black bars) and control (white bars) worms was calculated for 3 day, 4 day and 5 day old worms that received massed stimulation at a 204 second inter-stimulus interval. B) Differences between average tap withdrawal response magnitudes standardized over worm length across the 10 test taps between stimulated (black bars) and control (white bars) worms was calculated for 3 day, 4 day and 5 day old worms that received massed stimulation at a 10 second inter-stimulus interval.

The absence of any behavioral difference in the tap withdrawal response between massed control worms led us to hypothesize that the delivery of spaced stimulation was
required to produce significant changes in adult behavior. To investigate this hypothesis we
delivered a form of spaced stimulation shown not to produce 24 hour long-term memory in *C. elegans* (Beck and Rankin, 1997; Rose and Rankin, 2001). Worms were stimulated using a
spaced stimulation protocol at a 10 second inter-stimulus interval during L1 and were tested at
3 days, 4 days and 5 days of age. No behavioral difference was observed between stimulated
and control worms at 4 days (n = 27 for stimulated and control; p ≤ 0.5899) and 5 days of age
(n = 27 for stimulated; n = 26 for control; p ≤ 0.2981); however, 3 day old stimulated worms
exhibited a significant depression in the magnitude of the tap withdrawal response compared to
control worms (n = 30 for stimulated and control; p ≤ 0.0144; Figure 21).

![Figure 21. Effects of Spaced Stimulation at a 10 Second ISI on Adult *C. elegans* Behavior](image)

Differences between average tap withdrawal response magnitudes standardized over worm
length across the 10 test taps between stimulated (black bars) and control (white bars) worms
was calculated for 3 day, 4 day and 5 day old worms that received massed stimulation at a 204
second inter-stimulus interval.

To distinguish whether the significant depression observed in 3 day old worms that
received spaced stimulation at a 10 second inter-stimulus was similar to the significant
behavioral depression observed as a result of long-term habituation training or L1 spaced
stimulation at a 60 second inter-stimulus interval, we tested whether the depression was sensitive to reconsolidation blockade. Spaced stimulation was delivered at a 10 second inter-stimulus interval during L1 and 24 hours later worms were given 10 reminder taps at a 10 second inter-stimulus interval followed by 40 minutes of heat shock at 32 degrees centigrade. My preliminary results showed that the behavioral depression was observed in 3 day old worms that did not receive heat shock treatment (n = 19 for stimulated and control; p ≤ 0.0416; Figure 22B). Interestingly, the administration of heat shock following reminder treatment does not appear to affect the 3 day behavioral depression (n = 19 for stimulated and control; p ≤ 0.1708; Figure 22B). Thus, similar to the behavioral enhancement observed when spaced stimulation was delivered at a 60 second inter-stimulus interval, the behavioral depression observed when spaced stimulation was delivered at a 10 second inter-stimulus interval appears to be insensitive to reconsolidation blockade. Therefore, the behavioral depression at 3 days is unlike the 5 day behavioral depression that is observed as a result of long-term habituation training or L1 spaced stimulation at a 60 second inter-stimulus interval.
Figure 22. 5 Day Depressed Behavioral Response Appears to be Insensitive to Reconsolidation Blockade. A) Reconsolidation protocol, stimulated worms received 5 blocks of 20 taps and 10 taps for reminder both delivered at a 10 second inter-stimulus interval. Testing consisted of measuring the response to 10 taps at a 60 second inter-stimulus interval; B) Average response to tap over worm length during testing for control (white bars) and stimulated (black bars) worms that received reminder training followed by heat shock or no shock.
DISCUSSION

Although many studies have demonstrated significant changes in the nervous system and behavior of rodents reared in sensory enhanced environments, none have looked at whether these changes prolonged into adulthood. A few studies have demonstrated similar activity dependent plasticity effects in adult rodents exposed to sensory enhanced environments; however in these cases, rodents were only exposed to the enriched environment during their adulthood and were tested three to four weeks later. Thus none of the current studies have investigated the long-term effects that rearing in sensory enhanced environment produces on the nervous system and behavior.

The objective of this thesis was to investigate the long-term effects that an enhancement in sensory experience during development had on the biology and function of the nervous system. To accomplish this, I used *C. elegans* as a model system as it has a well characterized nervous system and a short developmental timeline. The results in experiment 1 and 2 demonstrated that spaced mechanical stimulation at a 60 second inter-stimulus interval delivered early in larval development (L1) produced significant long-term changes in the tap withdrawal behavioral response, GLR-1::GFP distribution and *glr-1* mRNA levels of adult worms: 3 day old worms exhibited a significant enhancement in the behavioral response, GLR-1::GFP distribution and *glr-1* mRNA levels compared to control worms, whereas 5 day old worms exhibited a significant depression of these three measures compared to control worms. In experiment 3, I demonstrated the presence of a critical period for the 3 day enhanced behavioral response and GLR-1::GFP distribution during L1, while no critical period was observed for the 5 day depressed behavioral response and GLR-1::GFP distribution. In experiment 4, I showed that the depressed behavioral response of 5 day old worms was
sensitive to reconsolidation blockade, whereas the 3 day enhanced behavioral response was insensitive to reconsolidation blockade. Finally, in experiment 5, I showed that the differences in the tap withdrawal behavior observed in L1 stimulated 3 day and 5 day old worms was dependent on the temporal and spatial pattern of stimulation delivered.

The different behavioral and molecular effects that early mechanosensory stimulation at a 60 second inter-stimulus produced in 3 day and 5 day old worms led me to hypothesize that separate cellular phenomenon produced the enhanced 3 day and depressed 5 day behaviors. Thus the enhanced behavior and increased distribution of GLR-1::GFP and glr-1 mRNA levels seen in 3 day old L1 stimulated worms may be independent from the depression of these three measures observed in 5 day old worms stimulated during L1.

LONG-TERM HABITUATION AT 5 DAYS

The depressed behavioral response observed in 5 day old worms that had received spaced stimulation at a 60 second inter-stimulus interval during L1, L2, or L4 shares many common features of long-term habituation. A number of criteria have been demonstrated to distinguish long-term habituation from other forms of response decrements. These criteria include, inter-stimulus interval (Beck and Rankin, 1997; Rose et al., 2002), temporal pattern of stimulation (spaced vs. massed), activation of gene transcription, and protein formation (Scharf et al., 2002), and the requirement of glutamate activity (Rose et al., 2003).

An important feature of spaced stimulation protocols that can influence their efficacy for long-term habituation is the rate at which stimuli are delivered. Davis (1970) reported that the delivery of spaced stimulation with a long inter-stimulus interval resulted in greater long-term habituation than delivery of spaced stimulation with a short inter-stimulus interval. Beck and Rankin (1997) showed that the formation of long-term habituation to the tap stimulus in C.
elegans was observed only when stimuli were delivered at a long inter-stimulus interval (i.e. 60 seconds). When spaced stimulation was delivered at a short inter-stimulus interval (i.e. 10 seconds) there was no induction of long-term memory for habituation. Similarly, in experiment 1, when I delivered spaced stimulation to C. elegans at a long inter-stimulus interval (i.e. 60 seconds) during LI, a significant depression in the magnitude of the behavioral response to the tap stimulus was observed in 5 day old worms; however, when spaced stimulation was delivered at a short inter-stimulus interval (i.e. 10 seconds) no difference in the magnitude of the behavioral response was observed between 5 day stimulated and control worms. These results demonstrate that only spaced stimulation delivered at a long inter-stimulus interval was able to produce a significant decrease in the magnitude of the behavioral response of 5 day old worms. Thus, the pattern of stimulation delivered during LI significantly affected the behavior of 5 day old worms.

Numerous studies have demonstrated that multiple stimulation trials are generally more effective for the production of long-term habituation when they are spaced apart rather than when they are massed together. This phenomenon is referred to as the trial spacing effect (Ebbinghaus, 1885; Rescorla, 1988; Latal, 1999). The trial spacing effect has been extensively studied at the behavioral level in a number of model systems. Carew et al. (1972) demonstrated that Aplysia is capable of greater long-term habituation for the siphon withdrawal and gill withdrawal response after multiple spaced training trials, than after a single massed training trial. In addition, multiple spaced stimulation trials delivered to Drosophila resulted in the formation of long-term memory to an odor avoidance task compared to flies stimulated with a single massed stimulation protocol (Tully et al., 1994). Furthermore, the delivery of spaced stimulation was necessary for C. elegans to exhibit long-term habituation to the tap stimulus; no long-term habituation was seen if the same number of habituating stimuli
delivered at the same inter-stimulus interval, were presented in a single training block (Beck and Rankin, 1997). In experiment 5, I demonstrated that stimulation delivered as a single block during L1 at a 10 second, 60 second or 204 second inter-stimulus interval did not produce any behavioral depression at 5 days. Therefore, similar to what has been seen in other systems, the 5 day behavioral depression was also dependent on the delivery of spaced stimulation.

The success of multiple spaced training sessions at long inter-stimulus intervals on the formation of long-term memory has been hypothesized to occur through the triggering of a molecular cascade by each training block that results in establishing long lasting cellular and synaptic changes that are required for memory encoding (Baliely and Chen, 1983; Castellucci et al., 1978). Abel et al. (1997) demonstrated using mice that the delivery of spaced stimulation produced an increase in calcium influx through receptor-associated ion channels in the hippocampus, in particular glutamate-receptor-associated-channels that activated a protein kinase cascade resulting in the activation of gene transcription factors. Protein kinase A (PKA) has been shown to be essential for this molecular cascade (Abel et al., 1997). Once PKA is activated (phosphorylated) it will activate itself (autophosphorylate) resulting in the activation of gene transcription factors, such as the cyclic AMP-response element binding protein (CREB; Yin et al., 1994) and the mitogen-activated protein kinase (MAPK; Abel et al., 1997). The activation of both CREB and MAPK results in the transcription of genes and protein formation that will likely result in permanent changes in the cell and at the synapse which are essential for memory encoding. Blocking AMPA type glutamate receptors during spaced training has been shown to result in the abolishment of long-term memory formation in C. elegans (Rose et al., 2003), an impairment of a reward-related instrumental task in rats (Hernandez et al., 2005) and an absence of the habituation of the gill withdrawal response in
Aplysia (Ezzedine and Glanzman, 2003). In my studies, I found that the 5 day depressed behavioral response was not observed when the activity of AMPA type glutamate receptors were blocked during the delivery of spaced stimulation. This suggested that each spaced stimulation block may be associated with an increase in calcium influx through AMPA type glutamate receptors. This increase in intracellular calcium levels would lead to changes in gene transcription and protein formation that would be associated with changes in behavior.

Massed stimulation or multiple spaced training sessions at a short inter-stimulus intervals have been shown to be not as effective as spaced stimulation at a long inter-stimulus interval for memory formation (Carew et al., 1972; Tully et al., 1994; Beck and Rankin, 1997). The delivery of spaced stimulation during L1 at a short inter-stimulus interval (10 seconds) or as a single massed block produced no depression in the behavioral response of 5 day old worms. Abel et al., (1997) demonstrated that when stimulation was delivered as single massed training trial the increase in intracellular calcium levels was insufficient to activate CREB and its downstream targets. In addition, Yin, Del Vecchi, Zhou and Tully (1995) showed that massed training was effective for long-term memory formation only when the activated form of CREB was induced. These results led us to hypothesize that the delivery of massed stimulation or spaced stimulation at a short inter-stimulus interval during L1 produced a change in the pattern of calcium currents that was insufficient to activate gene transcription factors and produce long-term cellular changes associated with memory formation. Therefore, only the delivery of multiple spaced training trials during L1 was sufficient to produce long-lasting biological changes at the synapse that are associated with the depressed behavioral response observed in 5 day old worms.

Previous studies have demonstrated that the formation of long-term memory is associated with a change in gene transcription and protein formation. Rose et al. (2003)
showed that the formation of 24 hour long-term memory in *C. elegans* was associated with a decrease in GLR-1::GFP distribution. In experiment 2, I showed that the 5 day depressed behavioral response seen in L1 stimulated worms was associated with an overall decrease in GLR-1::GFP distribution. Since the GLR-1::GFP construct did not specifically identify membrane-bound GLR-1 versus intracellular GLR-1, the overall decrease was not simply due to cytosolic internalization and re-packaging of GLR-1 for membrane insertion at a later time as has been seen in mammalian neurons (Malinow and Malenka, 2002). Rather it means that GLR-1 proteins have been removed and/or degraded from the cell process altogether and the overall expression levels are lower in stimulated worms. The fact that *glr-1* mRNA levels were also significantly lower in stimulated worms suggested that in addition to the removal and/or degradation of GLR-1 proteins from the cell process, a down regulation of *glr-1* gene transcription also occurred as a result of early spaced stimulation. Therefore, similar to long-term memory in *C. elegans*, the depressed behavioral response of 5 day old worms was associated with a decrease in overall GLR-1::GFP distribution.

It has been hypothesized that long-term depression (LTD) is the cellular correlate for long-term habituation (LTH), since the mechanisms of memory for LTH resemble those responsible for LTD of synapses in the mammalian hippocampus and cortex (Mulkey and Malenka, 1992; Mulkey et al., 1993; Kirkwood and Bear, 1994). The similarities between LTD and LTH are outlined in Table 3. These similarities suggest that an LTD-like synaptic mechanism may mediate the depressed behavioral response observed in 5 day old L1 stimulated worms.
Long-Term Depression | Long-Term Habituation
---|---
Produces a decreased synaptic response | Produces a decreased behavioral response
LTD expression requires low frequency electrical stimulation with robust changes requiring several stimuli | LTH expression requires a number of stimuli presented at a long inter-stimulus interval
Requires the presence of protein phosphatase | Requires the presence of protein phosphatase
Associated with a rapid internalization of AMPA receptors so there are fewer AMPA receptors at the synapse | Endocytosis of AMPA receptors is one mechanism hypothesized to occur as a result of long-term habituation training.
Ubiquitination of AMPA receptors is a molecular mechanism for the down regulation of AMPA receptors seen with LTD | Ubiquitination of GLR-1 has been demonstrated in *C. elegans* and is hypothesized to account for the decreased GLR-1 expression observed as a result of long-term habituation training.

**Table 3. Comparison Between LTD and LTH for Memory.**
The similarities in the effects, induction protocols and some of molecular factors involved in regulating LTD and LTH are outlined.

Gene transcription and protein synthesis have been shown to be associated with the consolidation of ‘new’ memories (Lee, Everitt and Thomas, 2004). Recently, it has been demonstrated that when a memory is recalled by an associated cue it returns to a labile state and is sensitive to some of the same initial consolidation factors. The inhibition of reconsolidation by pharmacological blockade of transcription or translation products abolished memory reconsolidation resulting in amnesia (Nader et al., 2000; Lee et al., 2004; Rose and Rankin, 2005). Thus the recall of a memory appears to place it into an active and labile state. Rose et al. (2005) demonstrated that the formation of long-term habituation in adult *C. elegans* was sensitive to reconsolidation blockade. Similarly, I found in experiment 4 that the delivery of heat shock following a reminder tap stimulus session 24 hours after initial spaced stimulation was sufficient to block the 5 day depressed behavioral response. Furthermore, the
sensitivity of the 5 day depressed response to reconsolidation blockade was still observed when I increased the duration of time between the delivery of the reminder taps and testing from 24 hours to 72 hours. This suggested that the depressed behavioral response observed in 5 day old worms was sensitive to reconsolidation blockade and that older memories, when retrieved return to a labile state and are subject to disruption, and require a protein-synthesis-dependent reconsolidation process

Reconsolidation has been suggested to involve mechanisms similar to the consolidation process; however the specific molecular mechanisms underlying reconsolidation events remain unknown. Nader et al. (2002) suggested that the retrieval of memories may destabilize the synaptic or cellular events associated with consolidation in such a way that they now have to be reconsolidated with new proteins. Rose and Rankin (2005) showed that the consolidation of new memories and reconsolidation of reactivated memories in C. elegans have been shown to require glutamate receptor activation. In addition, both consolidation and reconsolidation of these memories were associated with a downregulation of postsynaptic glutamate receptors (Rose and Rankin, 2005). In experiment 2 and 4, I found that the depressed behavioral response of L1 stimulated 5 day old worms, required glutamate activity, protein synthesis and was associated with a downregulation of postsynaptic glutamate receptors. It would be interesting to explore whether the reconsolidation of the reactivated memory was dependent on the same factors as the initial consolidation. This could be explored by investigating whether the blockade of glutamate activity by exposing worms to DNQX during reactivation abolished the depressed behavioral response of 5 day old worms and whether the total area of GLR-1::GFP was affected by reconsolidation blockade treatment. These experiments would enhance our understanding of the molecular mechanisms employed to reconsolidate reactivated memories that have been stored for long periods of time.
In experiment 3, I investigated whether there was a critical period for the depressed behavioral response of L1 stimulated 5 day old worms. I found that the depressed behavioral response of 5 day old worms was also observed when spaced stimulation was delivered during L1, L2 or L4. Interestingly, no 5 day behavioral depression was observed when spaced stimulation was delivered during L3. The absence of any behavioral effect when stimulation was delivered during L3 may reflect a biological change that is occurring at this developmental stage. There are significant differences between the structure of the larval and adult nervous system with respect to the tap withdrawal circuit. The birth of the interneurons AVM and PVM at 11.5 hours after egg lay (Driscoll and Kaplan, 1997), the birth of the PVD interneuron at 22.5 hours after egg lay (Driscoll and Kaplan, 1997), the wiring up of AVM to the tap withdrawal circuit between 32.5 – 37.5 hours after egg lay (Chalfie et al., 1985), and the addition of 56 neurons to the ventral motor nervous system during postembryonic larval development (Driscoll and Kaplan, 1997) are some of the biological differences between the larval and adult C. elegans nervous systems. Thus, L3 may reflect a transition period between the larval nervous system and the adult nervous system or may simply reflect a time when circuit rewiring lowers the sensitivity of the system to experience driven plasticity.

The results presented in this thesis demonstrated that the 5 day depressed behavioral response was dependent on the delivery of spaced stimulation at a long inter-stimulus interval. The absence of any behavioral depression in 5 day old glr-1 mutant worms and the requirement for glutamate activity for this behavioral depression to be observed suggests that calcium influx through glutamate receptors leads to the activation of gene transcription factors resulting in the behavioral depression seen in 5 day old worms. By changing the temporal and spatial pattern of stimulation delivered during L1, the influx of calcium is affected resulting in a change in calcium currents leading to different behavioral outcomes. In addition, the sensitivity of the 5
day depressed response to reconsolidation blockade demonstrates the requirement of gene transcription and protein formation for worms to exhibit the behavioral depression. The results presented in this thesis clearly demonstrate that the behavioral depression seen in L1 stimulated 5 day old worms depends on a number of the same criteria as learning and memory formation in other animal model systems.

3 DAY ENHANCED BEHAVIORAL RESPONSE IS A DEVELOPMENTAL EFFECT

Numerous studies have demonstrated that early experience is critical for regulating normal behavioral and nervous system development. Animals exposed to a deprivation of sensory experience exhibited significantly different behaviors as adults compared to animals raised in standard laboratory conditions. Monocular deprivation in kittens and Drosophila, have been shown to produce significant decrements in the nervous system structures responsible for mediating vision (Barth et al., 1997). The effects of deprivation become more pronounced as the intensity of sensory loss in the experimental paradigm increases. Rodents reared in sensory deprived environments (i.e. separated from their mothers and siblings) are developmentally delayed, exhibited significant decrements in the molecular properties of their nervous system and performed poorer in spatial learning tasks compared to rodents reared in standard laboratory environments (Rosenzweig et al., 1968; Turner and Greenough, 1985). The effects of sensory deprivation in C. elegans are similar to those reported for other model systems, and include a developmental delay that is accompanied by changes in adult behavior and the molecular properties of the nervous system (Rose et al., 2005). These studies demonstrate the detrimental effects sensory deprivation has on the behavior and nervous system in adults.
Conversely, an enhancement in sensory experience during development appears to produce the opposite effects that are associated with sensory deprivation. An enhancement in sensory experience seems to accelerate the development of the nervous system and enhance the behavior being investigated. Rats whose eye lids were sutured open two days prior to normal eyelid opening exhibited a precocial development of the spontaneous alternation behavior compared to rats whose eyes opened at the normal time (Dumas, 2004). In addition, in experiment 1, I demonstrated that an enhancement of early mechanosensory experience (i.e. spaced stimulation) produced an enhanced behavioral response at 3 days of age, compared to control worms. Thus, similar to rodents, early mehanosensory experience produced enhanced behavioral responses in adult *C. elegans*.

The effects of enriched environments have also been explored in higher order cognitive functions, such as learning and memory. Numerous studies have observed an improved performance in learning and memory tasks in mammals reared in enriched environments versus mammals reared in standard laboratory conditions (Whishaw et al., 1984; Tees et al., 1990; Morgensen, 1991; Janus et al, 1995; Fernandez-Teruel et al., 1997). It is hypothesized that this improvement in learning and memory is mediated by changes in the development of synapses and their plasticity. One mechanism implicated in synaptic plasticity in the mammalian brain is the trafficking of AMPA receptors (Song and Huganir, 2002). 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 Luscher and Frerkeing, 2001, Malinow and Malenka, 2002). Rose et al. (2005) showed that *C. elegans* raised in isolation exhibited a decrease in the total area of GLR-1::GFP. The decrease in GLR-1::GFP area was attributed to a reduction in mechanosensory stimulation experienced by worms raised in isolation.
plasticity of the *C. elegans* GLR-1 receptor during development was further explored by investigating the effects an enhancement in sensory experience would have on its expression. In experiment 2, I demonstrated that 3 day old worms expressed a significantly greater area of GLR-1::GFP and increased *glr-1* mRNA levels when exposed to an enhancement of mechanosensory stimulation in L1 compared to control worms. Thus, changing the amount of experience a worm is exposed to during development, produced significant changes in postsynaptic receptor expression, thereby affecting synaptic communication and behavior.

The effects of L1 stimulation in 3 day old worms shares some similarities to the effects observed with the induction of long-term potentiation (LTP). Three mechanisms have been extensively investigated in the expression of hippocampal LTP. These mechanisms include the increase in AMPA receptor insertion into the plasma membrane via activity dependent changes in AMPA receptor trafficking, an absence of presynaptic changes, and an increase in AMPA receptor phosphorylation, (for review see Malenka and Bear, 2004). Similar to the effects on hippocampal cells produced by LTP paradigms, the enhanced behavior observed in L1 stimulated 3 day old worms was positively correlated to an increase in the total area of GLR-1 distribution. In addition, L1 stimulation did not produce in any significant difference in the distribution of the presynaptic protein SNB-1. Thus, both LTP and early sensory enhancement produce significant increases in the distribution of AMPA receptors and an absence of presynaptic changes, suggesting the possibility that these two effects may be regulated through similar cellular mechanisms.

Sensitization is a form of nonassociative learning, where the animal exhibits an increased behavioral response due to the delivery of a strong, generally noxious stimulus. In *Aplysia* short-term and long term sensitization of the gill withdrawal and siphon withdrawal responses have been observed (Carew et al., 1972). The enhanced behavioral response and the
increased distribution of GLR-1::GFP expression and glr-1 mRNA levels observed in 3 day old worms that had received spaced stimulation during L1, led us to investigate whether this enhancement was a sensitized response due to early spaced stimulation. Using reconsolidation blockade I showed that the delivery of heat shock following a reminder session 24 hours after initial spaced stimulation did not affect the enhanced behavioral response of 3 day old worms. Thus, the enhancement was insensitive to reconsolidation blockade. One possibility to explain the insensitivity of the behavioral enhancement to reconsolidation blockade is that the enhanced behavior may reflect a developmental effect that acquires and stores environmental experiences differently from 'memory'. The acquisition and storing of new and retrieved memories has been shown to be associated with changes in gene transcription and protein formation (Beck and Rankin, 1995). Thus, a future experiment could investigate how inhibiting protein synthesis by administering heat shock during the intervals between spaced stimulation blocks affects the enhanced behavioral response of 3 day old L1 stimulated worms. Furthermore, Rose et al. (2005) found that reconsolidation blockade disrupted the downregulation of GLR-1::GFP that is shown to be associated with long-term memory formation. It would be interesting to see whether reconsolidation blockade affected the increased distribution of GLR-1::GFP seen in 3 day old L1 stimulated worms. Both of these experiments would enhance our hypothesis that the enhanced behavioral response seen in L1 stimulated worms represents a developmental effect for the earlier training.

In experiment 5, I investigated the role of inter-stimulus interval on the 3 day enhanced behavioral response. Similar to 5 day old worms, massed stimulation produced no difference in the magnitude of the behavioral responses between stimulated and control worms. Interestingly, spaced stimulation delivered at a short inter-stimulus interval (i.e. 10 seconds) produced a significant depression in the magnitude of the behavioral response of 3 day old
worms. These results were rather surprising, as it has been demonstrated that delivery of
spaced stimulation at short inter-stimulus intervals produced no long-term behavioral effects in
*C. elegans* (Beck and Rankin, 1997). In addition it has been hypothesized that stimulation at
short inter-stimulus intervals produced an insufficient increase in intracellular calcium levels to
activate gene transcription factors and produce long-term cellular and behavioral changes
(Abel et al., 1997). I investigated whether the significant depression in 3 day old worms that
received spaced stimulation at a 10 second inter-stimulus interval exhibited a similar sensitivity
to reconsolidation blockade as was observed in 5 day old worms that received spaced
stimulation at a 60 second inter-stimulus interval. Using reconsolidation blockade, I found that
the delivery of heat shock following a reminder session 24 hours after initial spaced
stimulation did not appear to affect the depressed behavioral response of 3 day old worms.
Thus, the depressed behavioral response observed in worms that received spaced stimulation at
a 10 second inter-stimulus interval appears to be insensitive to reconsolidation blockade,
suggesting that it may be regulated by different cellular phenomenon than the depressed
behavioral response observed in 5 day old worms that received spaced stimulation at a 60
second inter-stimulus interval.

During development an organism passes through a number of critical periods in which
a particular environmental experience is required in order for normal development to occur.
An absence or enhancement of this environmental experience can result in the expression of
abnormal behaviors. In experiment 3, I demonstrated the enhanced behavioral response and
increase in the total area of GLR-1::GFP was only observed when stimulation was delivered
during *L*1. Stimulation delivered later in larval development produced no difference in either
of these two measures between stimulated and control worms, demonstrating the presence of a
critical period for these effects early in larval development during *L*1.
Numerous studies have shown that the nervous system of *C. elegans* undergoes a number of biological changes early in larval development. During L1, additional cells are added to the nervous system of worms (Sulston and Horvitz, 1977), the synaptic output of some of the embryonic derived motor neurons are remodeled, such as the rewiring of the DD neurons (Jorgensen and Rankin, 1997), and pruning of the neuronal AIM axons occurs towards the end of L1 (Kage et al., 2005). In addition, there are changes in the expression patterns of molecules known to influence the nervous system during L1. The expression ZIG proteins are first detected (Aurelio, Hall and Hobert, 2002) during L1. ZIG proteins have been shown to play important roles in maintaining the organization of the mature nervous system. In addition, the axon guidance molecule UNC-6/netrin is expressed in the motor neurons (Wadsworth, Bhatt and Hedgecock, 1996) and the production of the neurotransmitter serotonin is first detected during L1 (Sze, Zhang, Li and Ruvkun, 2002). These studies demonstrate that the nervous system of *C. elegans* is undergoing a number of biological changes during L1 and suggests an increased sensitivity to early environmental experiences during this stage. Thus, the delivery of early mechanical stimulation during L1 may result in a change in a developmental process and produces the enhanced behavior and GLR-1::GFP distribution observed in L1 stimulated 3 day old worms.

The 3 day enhanced behavioral response observed in worms that had received spaced stimulation at a 60 second inter-stimulus interval appears to be a developmental effect that occurs due to the enhancement in early mechanosensory stimulation. Similar to the depressed behavioral response of 5 day old worms, the 3 day enhancement was dependent on glutamate activity and was associated with a positively correlating change in glr-1 mRNA levels and GLR-1::GFP distribution. Reconsolidation blockade treatment 24 hours after L1 stimulation did not affect the enhanced behavioral response of 3 day old worms. Changing the inter-
stimulus interval at which spaced stimulation was delivered during L1 (i.e. 10 seconds) resulted in the worms exhibiting a depressed behavioral response. Similar to the enhanced response of 3 day old worms stimulated at a 60 second inter-stimulus interval, the depressed behavioral response that was insensitive to reconsolidation blockade. The presence of a critical period during L1 for the enhanced behavior and GLR-1::GFP distribution demonstrate the presence of activity dependent plasticity early in *C. elegans* development, suggesting that similar to other animal model systems, environmental manipulations can produce significant changes in the development of the nervous system and behavior. The results demonstrate that the behavioral enhancement seen in L1 stimulated 3 day old worms is a developmental effect and is not dependent on all of the same criteria as the depressed behavioral response seen in L1 stimulated 5 day old worms.

TRANSITION BETWEEN ENHANCEMENT AND DEPRESSION AT 4 DAYS

It is interesting to note that no difference in the behavioral response between stimulated and control 4 day old worms was observed when spaced stimulation was delivered during any of the four larval stages and with any of the temporal or spatial patterns of stimulation delivered during L1. In addition, no significant differences in both GLR-1::GFP distribution or *glr-1* mRNA levels were observed between stimulated and control 4 day old worms.

There are two possibilities that may account for the absence of any behavioral or molecular effect in 4 day old worms that received an enhancement in mechanosensory stimulation during L1. The first possibility relates to the opposing behavioral and molecular properties observed between 3 day and 5 day old worms that received spaced stimulation during L1. The differences between L1 stimulated 3 day and 5 day old worms led us to hypothesize that these two effects are regulated by separate cellular phenomenon. Thus, the
enhancement observed in 3 day old worms may reflect a short lived developmental effect that is mediated by one cellular phenomenon whereas the 5 day depressed behavioral response is mediated by a second cellular phenomenon. In this case, the absence of any behavioral effect observed at 4 days may reflect the presence of a biological or functional transition period between the 3 day enhanced and 5 day depressed behavioral and molecular measures. The second possibility to account for the absence of any differences in the behavior and GLR-1::GFP distribution between stimulated and control 4 day old worms may be that the enhancement seen at 3 days reflects a short-lived "developmental" effect of early stimulation. This short-lived developmental effect overshadows the long-term habituation seen in 5 day olds and exhibits a graded decay over time. Thus by 4 days of age the enhanced behavior and GLR-1::GFP distribution have degraded and are no longer observed. To test this possibility, one would have to devise an experiment that would eliminate the 3 day enhanced behavioral response, while having no effect on the depressed 5 day depressed behavioral response.

CONCLUSIONS

The data presented in this thesis suggest that the 3 day enhancement and 5 day depression observed in *C. elegans* that received spaced stimulation during L1 may reflect different independent cellular processes. I have demonstrated that the depression seen in 5 day old L1 stimulated worms depends on a number of criteria used to describe long-term habituation, whereas the enhancement observed in 3 day old L1 stimulated worms does not. This suggests that the enhancement seen at 3 days may reflect a short lived developmental effect of early stimulation that is mediated by one cellular phenomenon, whereas the 5 day depressed behavioral response bears many similarities to long-term habituation and is mediated by a second cellular phenomenon.
The results presented in this thesis illustrate the effects that an enhancement in mechanosensory experience has on the behavior and nervous system of C. elegans; however there are a few limitations to the interpretations made from these results. The first is that I have only examined the effects that early mechanosensory experience has on a single behavioral task. Future experiments could explore the effects of mechanosensory enhancement on other well characterized C. elegans behaviors, such as adult learning and memory or worm length. Also, I have only examined the effects of mechanosensory enhancement in a single animal model system; there may be species specific differences in how molecules encode early environmental experiences. Furthermore, I have only explored the effects that mechanosensory enhancement has on a single subset of proteins (glutamate receptors and vesicles). There are a number of other proteins and molecules involved in producing these effects. Future experiments could explore the role of glr-1 trafficking proteins and other glutamate receptors in producing the adult behaviors observed in sensory enhanced worms. Also, it would be interesting to investigate whether early experience affects the synaptic distribution of GLR-1 and other glutamate receptors. Despite these limitations, the data presented in this thesis supports other investigations using different animal model systems that an enhancement of early sensory experience alters the biology of the nervous system and its function.

By using a simple animal model system with a short developmental timeline, I have contributed new insights into the long-term effects that an enhancement of early experience produces on the behavior and molecular properties of the nervous system. I reported evidence that early spaced mechanosensory stimulation at a long inter-stimulus interval produced a behavioral enhancement at 3 days followed by a depressed behavioral response at 5 days. The different parameters shown to regulate these behavioral effects supports the hypothesis that
separate cellular phenomenon are recruited to regulate the expression of the enhanced behavior at 3 days and the depressed behavior at 5 days. By understanding how early experience recruits different cellular mechanisms, we can further our current understanding of how early experiences are encoded in neurons to produce different adult behaviors.
FOOTNOTES

The work presented in this thesis will be submitted for publication as: CORRELATED
CHANGES IN BEHAVIOR AND GLUTAMATE RECEPTOR EXPRESSION AS A
RESULT OF EARLY STIMULATION IN CAENORHABDITIS ELEGANS. In Genes, Brain
and Behavior.


Figure 23. Dose Response Curves to 24 Hour Drug Exposure.
Wild-type N2 worms and glr-1(n2461) mutant worms were placed on individual agar plates with 0 μM (n = 6), 1.75 μM (n = 9), 17.5 μM (n = 9), 175 μM (n = 8), 1750 μM (n = 10) or 17500 μM (n = 6) NaOH or 0 μM (n = 20 & n = 21), 0.5 μM (n = 15 & n = 18), 5 μM (n = 17 & n = 20), 50 μM (n = 15 & n = 20), 500 μM (n = 19 & n = 19) or 5000 μM (n = 15 & n = 13) DNQX mixed in a 1:1 ratio with food. 23.5 to 24.5 hours later, a train of six consecutive taps at a 8.5 Hz was delivered to the plate and the reversal response recorded and displayed. Error bars show the SEM (Figure and text from Chen, S., pers. comm.).
APPENDIX 2

Below I have outlined a brief description of the methods and results for some of the data obtained during my time in the lab that was excluded from my thesis.

*cat-2(e1112)*

To investigate the role of the neurotransmitter dopamine during development, worms carrying a point mutation in the enzyme tyrosine hydroxylase were tested behaviorally. Tyrosine hydroxylase is encoded by the *cat-2* gene in *C. elegans* and is the enzyme that catalyzes the rate limiting step in the synthesis of dopamine. Spaced stimulation was delivered to worms carrying a mutation in *cat-2* [*cat-2(e1112)*] during L1 and worms were tested at 3 days, 4 days and 5 days of age. The results obtained showed a trend towards an enhancement in the tap withdrawal response of 3 day old worms (*n* = 11 for stimulated, *n* = 10 for control, *p* ≤ 0.0559), no difference in response magnitudes between stimulated and control 4 day old worms (*n* = 7 for stimulated, *n* = 6 for control, *p* ≤ 0.1992), and a significant depression in the tap withdrawal response of 5 day old worms (*n* = 10 for stimulated, *n* = 9 for control, *p* ≤ 0.0418; Figure 23). It was not surprising that the behavioral enhancement was observed in 3 day old *cat-2(e1112)* mutant worms, since previous studies have shown that *cat-2(e1112)* mutant worms exhibited wild type behaviors when raised in isolation (Rose et al., 2005). This result supports my hypothesis that the enhanced behavior at 3 days is a developmental effect that occurs due to an enhancement of early sensory experience. Unexpectedly, the 5 day behavioral depression was still observed in *cat-2(e1112)* L1 stimulated worms. This result was unexpected as the *cat-2* mutation has been shown to disrupt long-term memory formation (A. Giles, pers. comm.). This discrepancy may be due to the different biological states of the *C.*
*elegans* nervous system when spaced stimulation was delivered (i.e. spaced stimulation delivered at 4 days for LTM studies and L1 in my experiment).

![Graph showing behavioral response magnitudes across testing age](image)

**Figure 24. Spaced Stimulation Delivered During L1 on Adult *C. elegans* carrying a mutation in *cat*-2.** Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old *cat*-2 mutant worms. * = p ≤ 0.05

**lin-10(1510)**

LIN-10 is a PDZ protein that is homologous to the XMint11 protein found in the mammalian nervous system (Rongo et al., 1998). In *C. elegans*, the LIN-10 protein is involved in trafficking the GLR-1 receptor to the postsynaptic plasma membrane in the ventral nerve cord (Rongo et al., 1998). To investigate whether the increase in the total area of GLR-1::GFP was due to an increase in GLR-1 trafficking, I delivered spaced stimulation to worms carrying a mutation in *lin-10* [lin-10(1510); J. Kaplan] during L1 and tested worms behaviorally at 3 days, 4 days and 5 days of age. The results showed an absence of any behavioral difference between stimulated and control worms at 3 days (n = 12 for stimulated, n = 8 for control, p ≤ 0.1564), 4 days (n = 10 for stimulated, n = 6 for, p ≤ 0.4743), and 5 days of age (n = 5 for
stimulated, n = 3 for control, p ≤ 0.4255; Figure 24). In addition, I observed similar smaller average reversal magnitudes to those seen in L1 glr-1(n2461) mutant worms. This result further supports a developmental role for GLR-1. I was unable to obtain large sample sizes for 4 day and 5 day old worms due to the bag of worms phenotype associated with this mutation.

![Graph showing behavioral response magnitudes](image)

**Figure 25. Spaced Stimulation Delivered During L1 on Adult C. elegans carrying a mutation in lin-10.**

No significant difference in the magnitude of the behavioral response to a tap was observed between stimulated and control C. elegans at all three ages. Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old lin-10 mutant worms.

**nmr-1(ak4)**

The C. elegans nmr-1 gene encodes for the NR1 subunit of the NMDA receptor. Worms carrying a mutation in nmr-1 [nmr-1(ak4)] exhibited normal long-term memory (J. Rose, pers. comm.) and wild-type behaviors when raised in sensory deprived environments (Rose et al., 2005). To investigate whether nmr-1 played a role in the behavior of worms exposed to an enhancement of sensory experience during development, I delivered spaced stimulation during L1 to worms carrying a mutation in nmr-1 [nmr-1(ak4)]. The results showed that the delivery of spaced stimulation resulted in a depressed tap withdrawal response.
at 3 days (n = 29 for stimulated; n = 27 for control, p ≤ 0.0293), an enhanced tap withdrawal response at 4 days (n = 30 for stimulated; n = 28 control, p ≤ 0.0046), and no behavioral difference at 5 days of age (n = 29 for stimulated; n = 28 for control, p ≤ 0.5477).

Figure 26. Spaced Stimulation Delivered During L1 on Adult C. elegans carrying a mutation in nmr-1.
No significant difference in the magnitude of the behavioral response to a tap was observed between stimulated and control C. elegans at all three ages. Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old nmr-1 mutant worms. * = p ≤ 0.05; ** = p ≤ 0.01

The results observed in nmr-1 mutants in response to an enhancement in mechanosenory stimulation were rather surprising as previous work in our lab and in other labs have been unable to demonstrate any strong behavioral deficit associated with the nmr-1 mutation (Rose et al., 2005). One possibility to explain the surprising behavioral responses observed in L1 stimulated nmr-1(ak4) mutant worms is that additional genetic mutations had been acquired in our nmr-1(ak4) mutant strain. To explore this possibility I delivered spaced stimulation during L1 to a newly obtained nmr-1(ak4) mutant strain (A. Maricq). Worms were tested only at 3 days and their responses were compared to an unstimulated control group. In
addition, I delivered spaced stimulation to an *nmr-1* mutant which contained an non-integrated *nmr-1* rescue construct. The *nmr-1* rescue worms were stimulated during L1 and tested only at 3 days of age. The results showed that the delivery of spaced stimulation to worms carrying a mutation in *nmr-1* during L1 resulted in a trend towards an enhanced response in 3 day old stimulated *nmr-1* mutant worms compared to control *nmr-1* mutant worms (n = 6 for stimulated; n = 5 for control, p ≤ 0.0862). Thus *nmr-1* does not appear to play a developmental role. Interestingly, a behavioral enhancement was not observed in stimulated 3 day old *nmr-1* rescue worms (n = 5 for stimulated and control, p ≤ 0.6550). This may reflect a missing *nmr-1* regulatory region in the rescue construct.

**Figure 27. Spaced Stimulation Delivered During L1 on Adult *C. elegans* carrying a mutation in *nmr-1*.**

Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day old *nmr-1* mutant and *nmr-1* rescue worms.

**KP537 (*glr-1* rescue)**

In figure 7 I showed that the 3 day enhanced and 5 day depressed behavioral responses were not observed in *glr-1(n2461)* mutant worms. To support the role of *glr-1* in mediating these behaviors, I tested a *glr-1(n2461)* mutant strain of worms containing a non integrated *glr-
I rescue construct (A. Hart). *glr-1* rescue worms were given spaced stimulation during L1 and transferred to individual plates during L3. The results showed that the presence of the *glr-1* rescue construct produced a trend towards an enhancement at 3 days (n = 10 for stimulated, n = 11 for control, p ≤ 0.0973), no behavioral difference at 4 days (n = 6 for stimulated, n = 5 for control, p ≤ 0.9096) and no behavioral difference at 5 days (n = 11 for stimulated and control, p ≤ 0.9367; Figure 28).

Figure 28. Spaced Stimulation Delivered During L1 on Adult *C. elegans* carrying a *glr-1* rescue construct.

The presence of the *glr-1* rescue construct appears to rescue the 3 day behavioral enhancement, but does not appear to have any effect on the 5 day depressed response. Differences between average response magnitudes standardized over worm length across the 10 test taps between control (white bars) and stimulated (black bars) worms was calculated for 3 day, 4 day and 5 day old worms carrying a *glr-1* rescue construct.

These results suggest that the behavioral enhancement was rescued in the *glr-1* rescue strain, thus confirming the role of *glr-1* in mediating this enhanced behavioral phenotype. Unfortunately, the 5 day depressed behavioral response of L1 stimulated worms does not appear to be rescued in the *glr-1* rescue strain. These results, although disappointing, were not surprising, as previous attempts to rescue the absence of long-term memory observed in *glr-1*
mutants by using the *glr-1* rescue strain have been unsuccessful (J. Rose, pers. comm.). It is interesting to note that previous studies in our lab have shown that the *glr-1* rescue strain was able to rescue the developmental defects but not long-term memory defects observed in *glr-1* mutant worms (Rose et al., 2005). This finding further supports my hypothesis that the 3 day enhancement is a developmental effect and that the 5 day depressed response reflects the formation of long-term memory.

**Imaging of L3 stage and 3 Day Old Worms Stimulated at a 10s ISI**

The delivery of spaced stimulation at a 10 second inter-stimulus interval produced a depressed behavioral response at 3 days of age. To investigate whether this depressed behavioral response was associated with a decrease in the distribution of GLR-1::GFP, I quantified the total area of GLR-1::GFP in L3 stage and 3 day old worms that received spaced stimulation at a 10 second inter-stimulus interval during L1. GLR-1::GFP was not quantified in 4 day and 5 day old worms as no behavioral differences between stimulated and control worms were observed. My preliminary results showed that the delivery of spaced stimulation at a 10 second inter-stimulus interval during L1 produced a trend towards a decrease in the total area of GLR-1::GFP in stimulated worms at L3 (n = 10 for stimulated, n = 14 for control, p ≤ 0.3566) and at 3 days of age (n = 6 for stimulated, n = 3 for control, p ≤ 0.2339; Figure 29). These results are exciting as it suggests that by the changing of the temporal pattern of stimulation delivered during L1 significant changes in the molecular components of the *C. elegans* nervous system are produced.
Figure 29. Spaced Stimulation at a 10s ISI Delivered During L1 Altered GLR-1::GFP expression in *C. elegans*
Quantification of GLR-1::GFP postsynaptic expression on interneuron processes in the posterior ventral nerve cord of a worm receiving spaced stimulation during L1 and unstimulated control worms. A) Differences between total area of GLR-1::GFP expression standardized over worm length in image for L3 GLR-1::GFP worms. B) Difference between total area of GLR-1::GFP expression imaged between L1 stimulated and control 3 day old worms.