CRITICAL AND SENSITIVE PERIODS FOR REVERSING THE EFFECTS OF MECHANOSENSORY DEPRIVATION ON DEVELOPMENT IN CAENORHABDITIS ELEGANS

by

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ABSTRACT

Sensory experience at different stages during development can alter structures and functions of the nervous system in different ways suggesting that the timing of the occurring experience is critical. In these studies, I used the nematode *Caenorhabditis elegans* as a model organism to demonstrate the effects of deprivation of mechanosensory experience on behaviour and development; I identified critical and sensitive periods for reversing the detrimental effects of deprivation by introducing mechanosensory experience during development. Earlier studies found that worms reared in isolation, without the mechanosensory stimulation from conspecifics, respond significantly less to a mechanical tap stimulus and are significantly shorter in body length than worms raised in age-matched colonies. An examination of elements of the synapse between the mechanosensory neurons and the command interneurons showed that in isolate-raised worms, this synapse was weaker (fewer post-synaptic glutamate receptors and fewer pre-synaptic vesicles) than the synapses of worms raised in a colony condition. In this thesis, brief mechanical stimulation at any time during development reversed the effects of isolation on the behavioral response to tap and glutamate receptor expression suggesting there is no critical period for these two measures. In addition low levels of stimulation early in development (at the beginning of larval stage L1), but not later, rescued pre-synaptic vesicular marker *p mec-7::SNB-1::GFP* vesicle expression suggesting there is a period during which brief mechanosensory stimulation can reverse the effects of isolation very easily. Larger amounts of mechanosensory stimulation resulting from rearing worms in isolation and transferring them to a colony at the start of L1, L2 or L3 rescued the effects of isolation on body growth suggesting there is a critical period to reverse the
effects of isolation on worm length that ends during L3. These results suggest that
development of different systems follow different rules/time courses. Rescuing one
aspect of development will not necessarily reverse the total effects of isolation on the
developing organism. Different aspects of development will require varying amounts of
stimuli at varying time points in development, which would all need to be taken into
account to fully rescue the effects of deprivation on the organism.
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CHAPTER I
OVERVIEW

1.1 GENERAL INTRODUCTION

Many processes of development are modified by activity occurring in the maturing nervous system. Activity-dependent processes shape the final patterns and strengths of many of the synaptic connections in all nervous systems. These processes can be defined as biological modifications an organism undergoes due to the consequences of specific events it experiences during development. Such events may include interactions with diverse environmental factors including different levels of nutritional (Hoet and Hanson, 1999), tactile (Rose et al., 2005; Meaney, 2001; Schanberg and Field, 1987), thermal (Klose and Robertson, 2004), olfactory (Kay and Laurent, 1999), gustatory (Krimm and Hill, 1997) and/or socioeconomic factors (Perry, 2002).

Studies using different model systems have described a variety of developmental effects resulting from rearing animals in an enriched compared to a deprived environment. Studies have shown that rats raised in isolation, with minimal stimulation, have thinner visual cortices (Diamond et al., 1972), less dendritic material in visual cortical neurons (Volkmar and Greenough, 1972) and fewer synapses per neuron (Turner and Greenough, 1985) than rats raised in enriched environments. Different aspects of the brain (from mitosis to synaptogenesis and pruning) can be modified by experiences that occur at different time points in development. How experience sculpts the developing neural circuitry is one of the most intriguing questions in developmental neurobiology.
The experiments presented here using the nematode *Caenorhabditis elegans* demonstrate critical and sensitive periods for the influence of enriched versus deprived environments on behavior and body growth. Furthermore, using genetic tools available in *C. elegans* I have taken a closer look at these outcomes and their effects at the synapse.

### 1.1.1 ENVIRONMENTAL STIMULATION AND PLASTICITY

Historically, it was thought that the size, structure, and functional connectivity of the brain unfolded according to a genetic blueprint and that once formed, the nervous system was relatively stable in its structure. A more contemporary view acknowledges the contribution of *experience* to the appropriate formation of connections within the brain. An effective way to study the effects of *experience* on brain development is to examine the effects of enriched experiences versus deprived experiences. In other words, manipulating environmental conditions and then examining their resulting outcomes. Pioneers in this area of research, Hubel and Wiesel (1963), were among the first to study the effects of sensory deprivation on the brain, where they concluded that kittens deprived of visual experience during the period when it is maturing, have severely impaired visual ability. They concluded that there are some pre-wired cells in the brain; however, other cells are still developing and are being modified with early experience; and those cells that are pre-wired are sometimes not enough for normal functioning of some structures such as the eye.

In support of Hubel and Wiesel’s work, Rosenzweig and others (Rosenzweig *et al.*, 1972) conducted experiments on complex rearing of post weaning animals and showed that by altering an animal’s environment the morphological characteristics of its
brain were altered. Resulting changes in brain structure were dependent on how the animal interacted with its novel environment: if interaction was minimal, little change was observed in the animal's behaviour and subsequent changes noted in brain structures were also limited; however, if the animal engaged in new a repertoire of behaviors while interacting with the novel environment, many alterations in brain morphology resulted including increases in brain weight, cortical thickness, glial number, neuron size, dendritic branching, and the number of synapses per neuron (for a review, see Greenough and Chang, 1989). Similar findings have been seen in invertebrates. For example, *Drosophila melanogaster* reared in isolation showed 15% fewer Kenyon cell fibers in their mushroom bodies than flies reared in group conditions (Heisenberg, Heusipp, and Wanke, 1995). In all, through the use of animal models, we have been able to conclude that placing animals in different environments (complex or deprived) is sufficient to evoke significant neural responses (i.e Rosenzweig, Love, and Bennett, 1968; Rose *et al.*, 2005) and alter additional aspects of nervous system development.

### 1.1.2 CONSEQUENCES OF DEPRIVATION OF MECHANOSENSORY STIMULATION DURING DEVELOPMENT

Mechanosensation can be manifested in a variety of forms. In mammals, maternal deprivation is a form of mechanosensory deprivation because animals are separated from their mother where they encounter an absence of specific touch stimulation. Rodent mothers exhibit licking and grooming behaviors as a form of maternal nurturing. In invertebrates, tactile stimulation from conspecifics or interaction with objects in their environment is a source of mechanosensory stimulation. Mechansosensation can also be
experienced through certain types of environmental influences such as vibrations or physically induced mechanical stimulation. In mammals, since the mother normally comprises the prime source of environmental input early in development, she essentially determines the infant’s psychobiological state. This is important because survival of offspring is highly dependent on its ability to process sensory information throughout development while adapting to changing conditions.

While external factors like the environment do not alter our basic human genetic code they can produce permanent changes in the way genes are regulated and expressed (Thaler, 1994). Thus, because our genes are sculpted by our environment, one developing organism can be very different from another organism of the same species due to variations in the amount of sensory stimulation an animal experiences throughout development.

Studies with rodents have show that the amount of licking and/or grooming rat pups receive from their mothers can lead to individual differences in hypothalamic-pituitary-adrenal (HPA) responses to stress (Meaney, 2001). The HPA axis is the internal neuroendocrine system that responds to stress and results in production of corticosteroid hormones that affect the brain, the cardiovascular system, and other systems in getting the body ready for what is known as the "fight or flight" mechanism (Van Oers et al., 1998). Meaney (2001) showed low levels of licking/grooming are correlated with pups that are more fearful and who show higher physiological responses to stress than pups that experience high levels of maternal licking/grooming from their mothers. Touch/mechanosensory stimulation from mothers serves to dampen HPA activity in the offspring, protecting the animals against the highly catabolic effects of adrenal
glucocorticoids (hormones that regulate sugar and fat metabolism) (Schanberg and Field, 1987) during a period of rapid development. This might help explain why warm nurturing families tend to promote resistance to stress and tend to diminish vulnerability to stress-induced illness (Smith and Prior, 1995).

In addition to changes in HPA activity, high levels of maternal licking/grooming in rodents is correlated with increased GABA_A receptor binding and increased mRNA expression for NMDA receptor subunits (Meaney, 2001). As a result, brain derived neurotrophic factor (BDNF), a type of neurotrophic factor which regulates neuronal growth, survival and function during development, is enhanced by touch stimulation early in development and decreased by maternal deprivation (Zhang et al., 1997; Chao, Sakai, Ma, McEwen, 1998), as is the release of growth hormone (GH) (Schanberg et al., 1984).

Altered levels of early stimulation can have effects that extend beyond the nervous system. The pattern of response in neonatal (the first 4 months after birth) rats separated from their dam is strikingly similar to that observed in failure-to-thrive children (classified as Reactive Attachment Disorder of Infancy; RADI). Children with RADI experience a lack of attachment to their parents which is comparable to children who are deprived of maternal nurturing. Nevertheless, as in the rat pup model of maternal separation, children with RADI also exhibit slow growth rates, diminished secretion of growth hormone (GH), and impaired responsiveness to administered GH (Powell et al., 1967; Frasier and Rallison, 1972).

There is evidence from many different systems that early stimulation sculpts the synapses of the nervous system (Bonhoeffer & Schatz, 1998). Deprivation of stimulation
during rearing in a variety of model systems such as rats (Diamond, et al., 1966; Greenough 1975; Turner & Greenough, 1985; Sirevaag & Greenough, 1987), frogs (Cline, 1991) and birds (Rollenhagen & Bischof 1994) can produce very specific neural defects. Thus, there is a large amount of data to show that variations in mechanosensory stimulation during development have profound effects on biochemical and hormonal processes that control growth and development.

1.1.3 REVERSING THE EFFECTS OF MECHANOSENSORY DEPRIVATION ON DEVELOPMENT

The notion that environmental enrichment might work as a preventative therapy following severe isolation or mechanosensory deprivation was based on the hypothesis that stimulating plastic changes in the visual cortex during periods of substantial development ought to be beneficial in establishing normal functioning of the eye (Hubel and Wiesel, 1963). In fact, there is now considerable evidence that rearing animals in complex housing (i.e. with abundant sensory stimulation) can improve functional outcomes of many different types health conditions such as stokes and cerebral injuries in adult rats (for a review, see Schallert, Leasure and Kolb, 2000).

A number of animal studies have shown that the detrimental effects of maternal deprivation are blocked with artificial ‘stoking’ of the deprived pups. Stroking with a fine paintbrush is a manipulation that mimics the touch stimulation derived from maternal licking and/or grooming in rodents which is comparable to the effects of maternal nurturing. Since small variations in early stimulation can have large consequences on development, even brief touch stimulation was able to reverse at least some of the effects
of early deprivation in infants as shown in the following experimental models:
deliverance of gentle stroking during development can negate the effects of isolation in
rat pups (Schanberg and Field, 1987); and stoking premature infants (in incubators) for
15 minutes three times a day leads to significant weight gain and more mature
behavioural patterns (Schanberg and Field, 1987) compared to control groups who
received standard nursery care but no additional tactile stimuli. In addition, Lovic and
Fleming (2004) raised rat pups with and without their mothers and they found that as
adults, artificially reared rats (reared without their mothers) showed less pre-pulse
inhibition of the startle response, and more trials to criterion in several stages of an
attentional set shifting task. These artificially-reared rats that were provided with
'maternal-like licking' stimulation (stroking with a paint brush), during artificial rearing,
were not significantly different from mother-reared rats.

In all, these studies have shown that the detrimental effects of maternal
deprivation can be reversed by administering brief touch stimulation during development.
The work on the role of early stimulation has been replicated across phylogeny
suggesting evolutionary importance of early experience and maternal nurturing. One
critical aspect of reversing the detrimental effects of isolation is when the
mechanosensory stimulation is being delivered during development. The timing of this
experience is crucial.

1.1.4 CRITICAL & SENSITIVE PERIODS FOR REVERSING THE EFFECTS OF
ISOLATION ON DEVELOPMENT
Since not all aspects of an animal’s world are certain at birth, the details of an animal’s physical characteristics vary, as do their habits and their social conditions. To deal with this uncertainty, the CNS maintains the capacity to modify its connections based on interactions an animal has with its environment. It may be that a lack of stimulation at certain times during development can cause serious effects on a developing organism; or it may be that the amount of stimulation experienced by the organism can alter aspects of its development. Depending on the types of experiences, the neuronal plasticity of the developing nervous system allows it to customize its connections in individual animals.

Many of these connections pass through a highly plastic period called a “critical period,” which usually exists in the early years of life when the capacity for neural adjustment in response to experience is substantially greater than it is in adulthood. The term "critical period" is used to describe a fixed period (with an abrupt beginning and an end) of time in development when certain experiences have to occur in order for development to proceed normally (Hensch, 2004). If the right experiences do not happen during that prescribed time frame, critical aspects of development might be hampered. On the other hand, there may be “sensitive periods” which refers to periods (beginning and ending gradually) during development when an organism is particularly receptive to certain kinds of environmental experiences (Nordeen and Nordeen, 1990). It is important to make this distinction between critical and sensitive periods because some aspects of development may have a period in development where certain events must occur for the organism to develop “normally,” whereas other aspects of development may have a
period of development where the organism is susceptible to certain experiences but will not develop “abnormally” if that incident was not experienced.

Critical periods are an important factor in understanding how deprivation affects an organism since they denote whether there is a specific, finite time period during which experience can exert its effects on the system. For example, in order to reverse the effects of touch deprivation on the HPA response to stress in adult animals, the animal must experience touch stimulation during the 1st and 2nd postnatal days (Jutapakdeegul, Casalotti, Govitrapong, and Kotchabhakdi, 2003). During this period, touch stimulation induces a significant decrease (30-36%) in serum corticosteroid as compared to the unstimulated group. Furthermore, glucocorticoid receptor gene expression in the adult touch stimulated group was significantly increased in several brain areas such as the hippocampus (19-21%), frontal cortex (26-34%) and midbrain (15-24%) (Jutapakdeegul et al., 2003). These results indicate that changes in the levels of hormone secretion and gene expression due to touch stimulation are needed during these first two days of life to reverse the effects of touch deprivation on the HPA response to stress.

The concept of critical periods is also very important when devising treatment measures for deprived offspring. It will be crucial to treat offspring at an appropriate age because certain aspects of the CNS may have already been wired to respond in a certain manner, so that some forms of treatment may not have any lasting effects on the organism if treatment is administered after a critical period is already over. A crucial question concerns the mechanism that mediates these enduring parental influences on the health of offspring during these critical periods. The evidence above supports the hypothesis that the relationship between early life events and health in adulthood is
mediated by sensory stimulation and that variation in this relationship effects the
development of neural systems which underlie the expression of both behaviour and
endocrine responses.

1.2 CAENORHABDITIS ELEGANS AS A MODEL SYSTEM

The nematode *C. elegans* was first chosen to be a biological model system by
Sydney Brenner in 1974; in 2002 he won the Nobel Prize for his collaboration on
discoveries concerning genetic regulation of organ development and programmed cell
death. Of its total 959 cells, *C. elegans* has only 302 neurons with approximately 5000
chemical synapses, 600 gap junctions, and 2000 neurochemical junctions for which a
complete wiring diagram is available (White, Southgate, Thomson, and Brenner, 1986).
Moreover, the entire genome of this worm has been mapped and sequenced which leaves
researchers with a wealth of mutations available for analysis whose genetic expression
patterns and basic functions are known (Hodgkin, Horvitz, Jasny, and Kimble, 1998).
Consequently, strains with mutations in a specific gene expressed in identified cells can
be analyzed in order to determine the role(s) of a particular gene for a specific behaviour
(Rankin, 2002). Forty-four percent of 1880 human proteins studied have nematode
counterparts (Wheelan et al., 1999) making *C. elegans* a potentially powerful tool to aid
in the understanding of the function and interaction of human disease genes (Culetto and
Sattelle, 2000).
This self-fertilizing hermaphrodite worm has a very short generation time, reaching adulthood within four days (96 hours post fertilization), making them highly practical for developmental studies. The worm develops through a series of four larval stages (L1, L2, L3, L4) until it reaches the young adult (YAD) stage before it is a fully reproducible adult (96 hours after fertilization) (see Figure 1.1 below).

The nematodes are a deep branch within evolution of the animal kingdom. Evolutionarily, nematodes are considered to be placed at the base of the branch of the division between the insect and vertebrate lineage. *C. elegans* is an animal with cell types such as muscle cells and nerve cells of similar organization to the equivalent cells of vertebrates. By the time nematodes branched away from the rest of the animal kingdom, most of the basic mechanisms necessary for the development and functioning of a complex animal may have already evolved. Many biological mechanisms are so well conserved across the animal kingdom; hence some genes can functionally substitute for each other in experiments comparing *C. elegans* and vertebrates.

**Figure 1.0. Caenorhabditis elegans.** This image portrays a hermaphrodite adult worm, probably around 96 hours after fertilization (image adapted from www.wormbase.org).
Figure 1.1 Caenorhabditis elegans Developmental Timeline. This figure illustrates the life cycle of a hermaphroditic C. elegans nematode from fertilization to adulthood. The time (in hours) in this figure is represented for normal growth with an optimal rearing temperature of 20 degrees Celsius. An adult hermaphroditic worm lays a fertilized egg which undergoes embryogenesis and enters its first larval stage (L1) as it hatches out of its egg. The worm then passes through larval stages L2-L4 before it becomes a young adult at 60 hours after fertilization. The worm is a fully reproducible adult at 96 hours after fertilization. These larval stages are distinguished by different cellular modifications occurring between each of these larval stages (Figure adapted from www.eb.tuebingen.mpg.de/.../wurm_entw.html).

This model system is very useful for studying the effects of mechanosensory deprivation on development because young hatching C. elegans are completely independent and require no maternal care to develop and thrive in their environment. In addition, the worm is transparent which allows for visualization of the synapse using synaptic genes tagged with green fluorescent tagged (GFP) proteins, where gene expression changes can be seen in individual identified cells. Together with laser ablation
of identified cells this can allow an understanding of the role single identified neurons play in behavior.

Moreover, this model system is ideal for studies of plasticity since 80-90% of their synapses appear to be reproducible between animals; therefore, one worm is almost genetically identical to the next reproducing animal with very little variation (White et al., 1986). Since their synapses are reproducible it allows for an impressive model to test the influences of environmental factors because the biology from animal to animal is almost identical; therefore, the direct effects of environmental manipulations can be studied without the concern for the effects of unknown biological confounding variables. In addition, there are no complex dendritic arbours on C. elegans neurons, so unlike the mammalian system, the synapses in C. elegans are en passant meaning they occur when two processes run parallel to one another.

1.3 BEHAVIOURAL PARADIGM: TAP WITHDRAWAL RESPONSE

In the behaviour experiments of this thesis, the dependent variable quantified and examined was the magnitude of the initial tap withdrawal response. C. elegans have the ability to exhibit a reversal response following a mechanical tap stimulus that is administered to the side of its Petri dish (see Figure 2.2 for detail on apparatus). This tap withdrawal response shows habituation upon repeated tap presentations (Rankin, Chiba and Beck, 1990). Habituation is defined as a accumulative decrease in the strength 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).
Laser ablation studies have identified the neuronal circuit that underlies the tap withdrawal response (see Figure 1.2; Wicks and Rankin, 1995).

![Neural Circuit of the Tap withdrawal Response](image)

Figure 1.2 Neural Circuit of the Tap withdrawal Response. Rectangles represent the touch cells, circles represent the interneurons 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 (administered to the side of a Petri dish that the worm rests on) simultaneously activates both forward and backward 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).

The basic elements of the touch circuit in *C. elegans* are six touch sensory receptors, five pairs of interneurons, and 69 motor neurons. The touch circuit can be divided into two interconnected units: the neural circuit for anterior touch (head touch) and the neural
circuit for posterior touch (tail touch). For the experiments in this thesis, I am primarily concerned with the response to a mechanosensory tap. The worm resting on the agar in a Petri dish detects the mechanical tap stimulus to the side of the plate which simultaneously activates both the posterior and anterior touch circuits; the integration of the competing backwards and forward processes produces an overall backward reversal response (Wicks and Rankin, 1996).

Some of the elements of the adult touch circuit in C. elegans arise after embryogenesis. After the worm has hatched and has entered the first larval stage L1, the worm is capable of locomotion and responding to gentle head touch (Chalfie et al., 1985); however, it has a nervous system that is considerably different from that of an adult worm. Newly hatched L1 worms have only 2/3 of their adult nervous systems, for example, they have a touch circuit with only four touch receptors as opposed to six in the adult. In addition, the newly hatched L1 worm has only 22 of the total 76 ventral nerve cord motor neurons it has as an adult (Sulston and Horvitz, 1977). In the L1 larval stage of development, cell division begins again and new neurons differentiate, migrate and send out processes (Wood, 1988).

After hatching, C. elegans require 3 1/2 -4 days to reach egg-laying adulthood. During these four days the worms proceed through four developmental stages as described in section 1.2 (See Figure 1.1). The occurrence of such large changes in the neural circuit would seem to indicate that there should be correspondingly large changes in behaviour. Surprisingly, this apparently is not the case for both head and tail touch withdrawal responses, because both behaviours are observed at all larval stages (Chalfie et al., 1985) and show the same patterns of habituation at all developmental stages.
It is only when environmental influences alter the development process of the neural circuit that we see altered behavioural responses (Rose et al., 2005).

1.4 THE ROLE OF GLUTAMATE AT THE SYNAPSE IN C. ELEGANS

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). This excitatory amino acid acts on four subtypes of receptors: AMPA (α-amino-2-hydroxy-5-methylisoxazole-4-propionic acid), NMDA (N-methyl-D-aspartate), kainite and metabotropic receptors (mGluRs) (Bleakman and Lodge, 1998). AMPA and NMDA are widely distributed in the CNS (Conti and Weinberg, 1999).

Glutamate is believed to play an important role in development, neuronal survival, sensorimotor processing and certain neuropathological conditions. Glutamate is produced from either glucose, via the Kreb cycle, or glutamine. In mammals, it is synthesized by glial cells, stored in synaptic vesicles, released by calcium-dependent exocytosis, and removed by carrier mediated reuptake into the nerve terminals of neighboring glial cells (Rang et al., 1999).

With a relatively simple nervous system of 959 somatic cells (Culetto and Sattelle, 2000), C. elegans is a useful model system for studying the role of glutamate in development. Genetic analysis of the worm nervous system is made easy because techniques such as mutagenesis and gene disruption (reverse genetics) have successfully created mutant animals that have severe deficits in neurotransmitter release, synaptic
release or receptor function, yet are still viable and fertile animals (Bargmann and Kaplan, 1998).

Glutamate is believed to be involved in many behaviours in *C. elegans* including mechanosensory behaviour mediating responses to tactile simulation. In *C. elegans* there are 10 putative ionotropic glutamate receptor subunits that have been identified: eight non-NMDA (*glr-1* to *glr-8*) and two NMDA receptor subunits (*nmr-1* and *nmr-2*) found almost exclusively in the interneurons and motor neurons but not in muscle cells (Brockie *et al*., 2001). *glr-1* and *glr-2* are most similar to the rat AMPA receptor GluR1 subunit, displaying 56% and 53% sequence identities respectively (Brockie *et al*., 2001). *glr-3* to *glr-7* are non-NMDA receptors that are not as easily classified while *glr-8* appears to be a more divergent glutamate receptor (Brockie *et al*., 2001). *nmr-1* and *nmr-2* display 43% and 40% identity to the rat NR1 and NR2A NMDA receptor subunits respectively (Brockie *et al*., 2001). The interneurons that control locomotion and which are involved in the tap withdrawal response express many of these glutamate receptor subunits: AVA (*glr-1, glr-2, glr-4, glr-5, nmr-1, nmr-2*); AVB (*glr-1, glr-5*); AVD (*glr-1, glr-2, glr-5, nmr-1, nmr-2*); and PVC (*glr-1, glr-2, possibly glr-5, nmr-1, nmr-2*) (Brockie *et al*., 2001). Moreover, *eat-4* encodes a vesicular glutamate transporter (Bellocchio *et al*., 2000) expressed in the touch cells ALM, AVM and PLM (Lee *et al*., 1999). As both the pre-synaptic touch cells and post-synaptic interneurons contain glutamate receptors, glutamate is believed to be an important neurotransmitter in modulating *C. elegans* mechanosensation. In this thesis I will focus on the role of the *glr-1* receptor subunit in developmental plasticity.
The nematode *C. elegans* is known for dramatic responses to early developmental experience. At a very young age (during larval stage L1), the worm integrates different sensory inputs, such as pheromones, chemical cues from food and temperature, that can alter morphology, physiology, and behaviour. If there is sufficient food, or too high a density of conspecifics worms can go into a state of arrested development called a "Dauer" larva in which it can wait several weeks for better conditions. Less extreme changes resulting from experience have also been seen. Even though the nematode nervous system is composed of only 302 neurons, the animal has the flexibility to remodel itself in response to other sensory information. Rankin and colleagues (Rose et al., 2005) used a biologically relevant source of sensory information, the presence of conspecific individuals, to test whether deprivation of mechanosensory stimulation alters developmental processes. They compared animals raised in isolation to animal raised in colonies of 20-30 animals. Animals raised in small colonies were strikingly different from isolate reared animals as adults. Colony reared worms had a much larger initial tap withdrawal response to mechanical stimuli than animals raised in isolation. Colony growth also accelerated development, so that animals grew larger and began laying eggs about 4 hours earlier (at 70 hours of development) than isolated worms.

A higher level of mechanical stimulation in colonies, presumably because of collisions between animals, may explain the larger initial tap withdrawal response behaviour in colony reared worms. A brief exposure to mechanical stimulation in the
middle of larval development increased the size of the initial tap withdrawal response in isolate reared worms to the level seen after group-rearing, but did not change the behavior of colony reared animals. The AMPA-type glutamate receptor GLR-1 is required for this effect as the decreased initial response to the tap stimulus in worms raised in isolation was not observed in isolated raised worms carrying a mutation in glr-1 [glr-1(n2461)] (Appendix, Figure 4.2).

Rose et al (2005) found that worms carrying modified receptors made up of GLR-1 tagged to GFP (GLR-1::GFP) to allow visualization of glr-1 expression levels, showed higher levels of glr-1 in the ventral nerve cords of colony reared worms, when compared to isolate reared worms. Pre-synaptic changes were also quantified using a GFP tagged synaptobrevin-1 (pre-synaptic vesicular associated 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 colonies expressed significantly more SNB-1::GFP compared to worms raised in isolation (Rose et al., 2005). Thus, worms reared deprived of mechanosensory stimulation showed significantly lower expression levels of the genetic marker (GFP) in both pre and post-synaptic elements of the mechanosensory circuit. These results suggest that in isolate raised worms, the strength of the synapses in the mechanosensory circuit was weaker than the same synapses in colony raised worms. These experiments are the first to demonstrate that different types of environmental experience can alter gene expression in the nervous system leading to changes in behaviour and development in C. elegans. In addition these results suggest the importance of mechanosensory stimulation during C. elegans development for normal synaptic formation in the tap withdrawal circuit.
1.5.2. BODY SIZE

The effects of isolation on the mechanosensory circuit and on body growth appeared to be mediated by different genetic pathways. Brief mechanical stimulation introduced in development did not rescue the effects of isolation on development and body size, although mechanosensory neurons play a role in the response. Worms carrying a mutation in \textit{glr-1} \([\textit{glr-1}(n2461)]\), showed the effects of isolation on the initial response to tap, but did not differ from wild-type worms in the effect of isolation on worm length (Appendix, Figure 4.2) suggesting that either touch cells are not important for the effects of isolation on worm length, or that the touch receptors use a non-GLR-1 pathway to affect size. Rose \textit{et al.} (2005) tested this hypothesis using a genetic strain \textit{mec-4}, a gene that codes for an amiloride-sensitive sodium channel that is expressed in the six touch sensory neurons; thus \textit{mec-4} worms are touch insensitive (Driscoll and Chalifie, 1991). \textit{mec} mutants with dysfunctional mechanoreceptors reared in isolation showed no difference in worm length compared to \textit{mec} mutants reared in colony condition (Appendix, Figure 4.3); thus the effects of isolation on worm length must be mediated through mechanosensory neurons (Rose \textit{et al.}, 2005) and the effects of isolation may have been caused by a lack of activation of the touch cells.

Moreover, in a paper investigating the effects of chemosensory deprivation on \textit{C. elegans}, Fujiwara \textit{et al.} (2002) showed that genetic abnormalities in the worm’s chemosensory organs decreased sensory input and led to decreased body size. This decrease in body size was mediated by \textit{EGL-4}, a gene that encodes a cGMP-dependent protein kinase that acts via the TGF-\textit{beta} signaling pathway. Although Fujiwara \textit{et al.}
(2002) showed that EGL-4 is activated by sensory input to regulate size, it is still not clear how is does this. Rose et al. (2005) showed that isolate reared egl-4 worms still show the effects of isolation on the initial response to tap; but both colony and isolate reared egl-4 worms are the same size at 4 days of age (Appendix, Figure 4.3).

Together, this data dissociates the effects of isolation on a behavior and on worm size into two separate genetic pathways dependent on touch cell activity. Both are mediated through the touch cells; however, behaviour is regulated by glr-1 and body size is regulated by egl-4. This supports the hypothesis that the effects of isolation on behaviour and on body growth are independent effects of developing in an environment with impoverished sensory input.

1.6 THESIS OBJECTIVES

In the current thesis, I have expanded on the results reported founded by Rose et al. (2005). Rose et al. (2005) demonstrated the worms reared in isolation have significantly smaller initial tap withdrawal response, express significantly less post-synaptic marker GLR-1::GFP and pre-synaptic marker pmec-7::SNB-1::GFP, and are significantly shorter in worm length compared to worms reared in a colony of 20-30 animals. They also showed brief mechanosensory stimulation delivered in the middle of larval development (larval stage L3) can reverse the effects of isolation on the initial tap withdrawal response and on post-synaptic marker GLR-1::GFP; however, they did not see in any differences in pre-synaptic pmec-7::SNB-1::GFP or worm length. I further explored these results to investigate whether there are critical or sensitive periods during development and/or if there are critical levels of stimulation required to reverse the
detrimental effects of mechanosensory isolation on the behavioral response to a mechanical tap, on post-synaptic glutamate receptor and pre-synaptic synaptobrevin expression in the worm, and on worm length.

I hypothesized that mechanosensory stimulation delivered at any stage of development will reverse the effects of isolation on the initial tap withdrawal response. With the same amount of mechanosensory stimulation, I hypothesized I would be able to reverse the effects of isolation on glr-1 glutamate receptors expression using the GLR-1::GFP construct expressed along the ventral nerve cord of the worm. On the other hand, since Rose et al. (2005) found that there was significantly less synaptobrevin expressed in the sensory neurons in worms raised in isolation, and that this was not rescued by brief mechanosensory stimulation in L3, I hypothesized that reversing the effects of isolation on pre-synaptic vesicle marker mec-7::SNB-1::GFP would require higher levels of stimulation than reversing GLR-1::GFP. Moreover, since brief mechanosensory stimulation delivered in the middle of larval development (L3) was not sufficient to reverse the effects of isolation on worm length, I hypothesized that it would also require greater levels of mechanosensory stimulation to rescue worm length in isolated worms.

In general, I hypothesized that all the (four) effects of isolation I investigated would be reversed with sufficient amounts of mechanosensory stimulation during development; there would be critical amounts of stimulation required for reversing different aspects of isolation; and that this investigation of critical periods to reverse the effects of isolation on development will help provide insight to understand the effects of early deprivation in other organisms including human infants, which may aid to produce treatment programs for human premature infants that suffer early sensory deprivation.
CHAPTER II

METHODS

2.1 SUBJECTS

A total of 1613 C. elegans were used in these experiments of which 1346 were
wild type (Bristol N2) originally obtained from the Caenorhabditis elegans Genetic
Center). To quantify changes of glutamate at the synapse using fluorescence confocal
microscopy we used two genetically engineered strains of worms. Post-synaptic changes
at the synapse were visualized using 127 GLR-1::GFP C. elegans. GLR-1::GFP is a
strain of worms that contains a chimeric receptor made up of GLR-1 tagged with the
green fluorescent protein (GFP) (Rongo and Kaplan, 1999). GLR-1::GFP was expressed
in the interneurons under the control of the glr-1 promoter and is expressed in clusters
along the ventral nerve cord of the worm. 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). Using 140 mec-
7::SNB-1::GFP C. elegans, we visualized pre-synaptic changes of GFP expression of the
gene snb-1 (synaptobrevin). snb-1 is a vesicular associated membrane protein (VAMP)
which marks a protein called synaptobrevin that plays a role in regulating vesicle fusion
at the synaptic terminal. In this construct, the snb-1 gene was fused to GFP and was
expressed under the control of the mec-7 promoter which targets this GFP expression to
the six mechanosensory neurons of the tap withdrawal circuit (Nonet, 1999).
2.2 REARING CONDITIONS

The following conditions were used for behavioural testing, body size analysis and confocal imaging: a control condition with isolate and colony worms that were reared un-disturbed until testing day and an experimental (also called stimulated) condition with the same number of isolate and colony worms as the control conditions. The only difference between the control and experimental group was that the experimental groups received mechanosensory stimulation at a particular point in time during development. All groups are tested at the adult age of 4 days (96 hours after fertilization).

For both behavioural testing and fluorescence imaging, there were 4 experimental groups in total (2 control and 2 experimental); the 2 control groups included an un-stimulated colony of approximately 30 worms and 20 un-stimulated individual isolated worms. The 2 experimental groups included a stimulated colony (that had been stimulated at a specific stage during larval development) and 20 stimulated isolates (that had been stimulated at a specific stage during development). The stimulated colony condition was included as an experimental control to control for any variations in the control condition. I hypothesized that there would be no difference between colony raised and stimulated colony worms. For the body size analysis experiment, there were only 3 groups, a control colony, control isolated worms and one experimental group that was either a transferred isolate group or a transferred colony group depending on the experiment (which is clarified in a later section).

2.2.1 CONTROL CONDITION
Worms were reared on Petri plates that were 60mm in diameter and 15mm deep, filled with 10mL of nematode growth medium (NGM) agar and seeded with one circular drop of *E. coli* (strain OP50) (Brenner, 1974) in the middle of the plate. Colony-raised worms were produced by allowing an adult hermaphrodite *C. elegans* to lay between 20 to 30 eggs then removing the adults so that only the age matched eggs remained on the plate. Isolate-raised worms were produced by allowing 1 adult hermaphrodite worm to lay one egg, then by removing the adult worm and any extra eggs leaving a single egg on the plate. To protect the worms from extraneous stimulation, all plates were then placed in a foam or cotton-lined box and placed in a 20+/- 0.2 degrees Celsius incubator. Plates were not touched until testing (96 hours later).

2.2.2 EXPERIMENTAL CONDITION

In experiments with a stimulated isolate and stimulated colony condition, worms were handled as described above for both the colony and isolate condition; however, at the start of specific larval stage of development (L1, L2, L3, L4, or YAD) they were removed from the incubator and given a brief period of mechanosensory stimulation. One box in the incubator was labeled “control” which contained 20 isolate plates and 2 colony plates and another box was labeled “simulated” experimental group which also contained 20 isolate plates and 2 colony plates. To deliver the same amount of stimulation to all the colony and isolate worms simultaneously, the stimulated experimental box was removed from the incubator and 30 tactile stimuli were delivered by dropping the box onto a table top from a height approximately 5 cm 30 times as a 10 second interstimulus interval (ISI)
Following this brief periods of stimulation, the box containing the worm was returned to the incubator until testing at 4 days of age.

**Figure 2.1** Box Drop Method for Mechanosensory Stimulation. This is a method to administer simultaneous mechanosensory stimulation to worms of the same age. Experimental groups (stimulated colony and stimulated isolate worms) were placed in a plastic Tupperware box lined with foam at hour 0 and were placed in a 20 degree Celsius incubator. The box was then removed from the incubator at the start of one of the larval stages (L1, L2, L3, L4, or YAD) at which the box is dropped 5 cm off a table top 30 times at a 10 second ISI. The box was then returned to the incubator and left there until testing day (4 days of age-96 hours after fertilization).

**2.3 BEHAVIORAL TESTING AND APPARATUS**

For behavioral testing, a platinum wire pick was used to transfer worms from the plate that they were grown on to a test plate without any *E. coli*. On test day (four days of age or 96-100 hours after eggs were laid), the test plate was placed in a plate holder that is attached to a micromanipulator, resting on the stage of the stereomicroscope (Leica Wild, Model M3Z). After two unrecorded minutes, the stage lamp was turned on and a single tap is delivered to the side of the plate via a copper rod driven by a Grass S88 stimulator and recorded using a video camera (Panasonic Digital 5100), connected to a VCR (Panasonic AG1960), and television monitor (NEC PM-1271A) (Figure 2.2). Each mechanical tap exerted approximately 1 – 2 Newton’s of force onto the plate. The date and time were displayed on the monitor via a time-date generator (Panasonic WJ-810) to
ensure taps were manually delivered accurately. All behavioural testing is done in a temperature and humidity controlled room set at 20 degrees Celsius and 40% humidity. These were optimal conditions for these assays.

2.3.1 TRANSFER PROTOCOL FOR WORM LENGTH ANALYSIS

Experiments in which I investigated the effects of mechanosensory deprivation on worm body size, I used two techniques: mechanical stimulation using the box drop technique as described above, and the transfer protocol. In the transfer protocol worms
received higher levels of mechanosensory stimulation for a longer period of time. This alternative method consisted of the “isolate to colony transfer” and the “colony to isolate transfer” protocol in which I varied the duration of isolation. As illustrated below in Figure 2.3, the isolate to colony transfer experiments consisted of worms that were raised in isolation and then transferred (at L1, L2, L3, L4 or YAD) to a single colony plate (composed of all the isolate worms) and then measured at four days of age (adult) protocol.

Figure 2.3 Transfer Method to Investigate Critical Periods to Reverse Effects of Isolation body size in C. elegans. The top portion of the figure illustrates the time line for worms reared in isolation and transferred using a platinum wire pick to a colony condition. The bottom portion of the graph is worms reared in a colony condition and then transferred to individual isolate conditions. This technique was adapted so that worms would receive greater amounts of mechanosensory stimulation throughout development instead of introducing brief mechanosensory stimulation using the box drop technique.
This allowed the isolated worms to receive mechanosensory stimulation on development from conspecifics over varying times of development. I also reversed this method where I reared the worms in a colony and transferred them to individual isolate plates (at a particular larval stage) in the colony to isolate transfer. This was done to see if isolation had any effect after an animal has already been developing normally.

Because there was some variability in the raw scores of wild type N2 worms across experiments as a result of changes in environmental conditions, it is important to point out that in each experiment for mechanical stimulation at each larval stage, all groups were run at the same time. Across larval stages, the effect of isolation on the response to a mechanical was consistent (isolates responded significantly less to a tap stimulus compared to colony reared worms). Since the colony/isolate effect was easily disrupted (i.e. if the worm’s experienced even very low levels of vibration during development the effect was lost) we only used experiments in which there was a significant difference between the colony and isolate group.

2.4 SCORING DATA

After recording the initial responses to the tap for the five experimental conditions, the reversal response magnitude (i.e. the distance the worm swam backwards in response to the mechanical tap delivered to the side of the Petri plate) was scored using a stop-frame video analysis. All scoring was done using sheets of acetate and a permanent fine tip marker. Before scoring the reversal for each worm, a tracing is made of the length of the worm as a measure of body size. Worm length was calculated by measuring the worm from the tip of the tail to the tip of the head. To score a reversal we
used the frame by frame VCR function and started with tracing a dotted lined body trace from the frame just before the tap is delivered. Then a solid line representing the actual reversal from the frame right after the tap is drawn capturing the length of the reversal within one second after the tap is delivered until the worm stops reversing. The criterion for scoring the videotaped data is divided into five different categories. A “reversal” to tap was defined as a worm swimming backward in response to the tap within one second of delivery of the tap. A “double reversal” was defined as a worm that has reversed more than once within 1 second of the tap (i.e. it may pause for a short period or swim forward but then swim backward all within 1 second after the tap). A “pause” was denoted when the worm does nothing for more than 1 second after the tap has been delivered. “No response” was used when the worm continues doing what it was doing before the tap. Finally, “already reversing” is used when the worm was already reversing when the tap was delivered; this data point was counted as a missed data point because all the data points accumulated should only consist of delivering the tap to worms swimming forward. The acetates containing these tracings are scanned (UMAX Astra 2100U) into a Macintosh computer using either DeskScan II or VistaImage software and measured using NIH Image software and then entered in Statview 4.5 for statistical analysis.

2.5 STATISTICAL DATA ANALYSES

In all experiments, statistical analyses was done using Statview 4.5 and by using a univariate (one-way) analysis of variance (ANOVA). This statistical technique allows us to analyze the difference between any number of sample means unlike the t-test which imposes restrictions on the number of means you could analyze at one time. If an
ANOVA test shows a significant p value, then it tell us the at least one of the means in all the groups that are being tested is different from another one mean. To test which means are different from each other, we ran a post-hoc analyses using Fisher’s planned least significant difference (PLSD).

Since we had planned comparisons developed before I ran the experiments, our experimental question was not “does the data provide convincing evidence that the means are not all identical.” Because our experimental questions were more focused, “were colony worms different from isolate reared worms, and were stimulated isolate worms different from isolates,” they were answered by multiple comparison tests (post tests) using the Fisher’s PLSD. In this case, we were able to safely ignore the overall ANOVA results and jump right to the post-hoc test results; nevertheless, since multiple comparison calculations all use the mean-square result from the ANOVA table, the post-hoc tests still require that the ANOVA table to be computed. Therefore an overall ANOVA was still calculated and still reported in the result; but post-hoc tests were done regardless of whether the ANOVA was significant.

2.6 CONFOCAL MICROSCOPY AND GREEN FLUORESCENCE PROTIEN (GFP) QUANTIFICATION

Investigating the effects of mechanosensory deprivation at the synapse was analyzed using confocal microscopy. At four days of age, the worms were removed from the incubator and prepared for imaging. Worms were placed in a 4-chambered Lab-Tek sterile slide in a drop of 10microL of 2,3-butanedione monoxide (BDM) for paralysis. Images were obtained using the BioRad Radiance Plus confocal on an inverted Zeiss-
Axiovert with DIC Optics (Bio-Rad) microscope equipped with a Krypton/Argon laser. GFP will be excited using a 488nm wavelength laser setting with the emitted light collected by passing through a ~510-550nm bandpass filter. The images collected were quantified using Image J 1.33 software. Optical sections were collected at ~0.4 μm intervals using a 63x oil lens.

Since post-synaptic \textit{glr-1} glutamate receptor marker GLR-1::GFP is expressed along the ventral nerve cord, images were collected along the posterior portion of the ventral nerve cord from the tail to the vulva. Since I used 63x magnification, the whole worm did not fit in a single image; therefore, a total of three images were taken from the tail up to vulva which were then quantified and added up to a single value per worm. Images of GLR-1::GFP expression were composed of 15-20 optical sections for each ventral nerve cord segment. The total stacks of images were then compiled into a single projection image in Image J 1.33. Collecting images for GLR-1::GFP were consistently acquired using the same microscope settings: gain=\~29; iris=\~4.0, laser=\~49%. The GFP expression in the ventral nerve cord is uniform in thickness, therefore the total area of GFP expression was measured (Rose \textit{et al.}, 2003).

Pre-synaptic vesicle marker \textit{pme}-7::SNB-1::GFP expressed GFP in the touch sensory neurons because it was a construct created under a \textit{mec}-7 promoter. Since, SNB-1::GFP appeared as one to three clustered bright spots along the ventral nerve cord, area measurements were used to quantify GFP expression (Rose \textit{et al.}, 2003). A single image comprised of 10-20 optical sections was collected from the region just posterior to the vulva of the worm. These images were captured at the same microscope settings that were: gain=\~39, iris=\~4.0, laser=\~50%. Using Image J 1.33 to quantify these images I
used a threshold adjustment which produced high-contrast images in black and white to allow for viewing of faint GFP. Area measurements for each region of GFP expression were calculated by outlining the GFP expressing region and using the area measure function in Image J 1.33. A researcher blind to the treatment groups measured the GFP expression in the images using NIH Image 1.61 and measurements were entered into Statview 4.5 for statistical analyses.
CHAPTER III

EXPERIMENTAL RESULTS

3.1 EXPERIMENT #1

Brief mechanosensory stimulation reverses the effects of isolation on the behavioural response to tap at each larval stage during development suggesting there is no critical/sensitive period.

Since various findings have suggested that certain developmental events have a critical or sensitive period for their expression, it is possible that *C. elegans* have a period during which mechanosensory stimulation is necessary for the mechanosensory system/circuit to develop normally. When worms first hatch, they do not have the same nervous system as an adult worm (for details on the distinction refer to Section 1.3). This may provide support why isolation during development lead to decreased mechanosensory responses, because the period of isolation may have been preventing the mechanosensory system to develop normally since it was missing mechanosensory experience.

Previous experiments conducted by Rose *et al.* (2005) showed that worms that developing under isolated conditions respond to tap with a significantly smaller response to tap than worms that developed in the presence of other worms (colony condition). The hypothesis is that the lack of mechanical stimulation from contact between conspecifics led to a decrease in responsiveness to mechanical stimulation. Rose *et al.* (2005) also showed that administration of brief mechanosensory stimulation (30 box drops at a 10s ISI) given in the middle of larval development (larval stage L3) to worms raised in
isolation and then tested at adulthood can reverse the behavioral effects of isolation on the response to single tap. This experiment suggested that introducing mechanical stimulation during the larval development can rescue the effects of deprivation if the stimulation is introduced during L3. The first experiment in this thesis was to determine whether introducing mechanical stimulation during other stages of development could reverse the effects of isolation and to see whether there was a critical or sensitive periods during development to reverse the detrimental effects of mechanosensory deprivation on the initial tap withdrawal response.

To test this hypothesis, brief mechanical stimulation (30 box drops at a 10s ISI) was delivered (at the beginning of a specified larval stage, L1, L2, L3, L4 or YAD) to worms raised in colony and isolate conditions and then tested at adulthood for their behavioural response to tap. Five different experiments were conducted, each consisting of introducing mechanosensory stimulation at the start of one of the 5 larval stages. In each experiment, there were three groups of worms, a control colony raised, a control isolate raised, and a stimulated isolate raised group. In pilot experiments, there was never an effect for the stimulated colony group so in order to increase my numbers for the other groups, I stopped running that group and I have omitted them from the analyses. The stimulated isolate group received 30 box drops at a 10s ISI at a start of a specified larval stage (Figure 3.1 & Table 1 summarizes the results for behavioural testing).

In all experiments, there were significant differences in the initial response to tap between control colony and control isolate reared worms as expected from Rose et al. (2005); worms reared in isolation responded significantly less to the mechanical tap stimulus compared to worms reared in a colony. Fisher’s PLSD post-hoc comparisons
showed that both colony raised and stimulated isolated raised worms responded with significantly larger reversals to the mechanical tap stimulus compared to control isolate worms in each experiment. Specifically, when introducing brief mechanosensory stimulation during L1 and then testing the response to tap at four days of age, stimulated isolated (n=15) responded with significantly larger reversals compared to control isolates (n=16) and were no different from control colony (n=16) reared worms; L1 experiment, F (2, 44)=3.7, p<0.03. Introducing stimulation at the beginning of L2 also rescued the response to tap as stimulated isolates (n=19) responded had significantly larger responses to tap compared to isolates (n=17) but were no different compared to responses of the control colony group (n=19); L2 experiment, F (2, 52)=4.3, p<0.02 Introducing stimulation at the beginning of L3 also reversed the effects of isolation as stimulated isolates (n=40) has significantly larger responses to tap compared to isolates (n=34) and were no different from the responses to tap from colony worms (n=43); L3 experiment, F (2, 114)=3.04, p<0.05. Introducing brief stimulation at the start of L4 also reversed the effect of isolation on response to tap in isolated worms as stimulated isolates (n=17) had significantly greater responses to tap compared to isolate (n=17) and were no different from colony (n=17); L4 experiment, F (2, 48)=2.8, p<0.07. Finally, introducing brief mechanosensory stimulation at the start of YAD (young adult) larval stage also reversed the effects of isolation on the response to tap as stimulated isolates (n=34) had significantly larger responses to tap compared to isolates (n=19) and were no different from responses to tap from colony reared worms (n=23); YAD experiments, F (2, 73)=2.8, p<0.07. In all, brief amount mechanical stimulation (30 box drops at a 10s ISI) during development rescued the effects of mechanosensory deprivation on the initial tap
withdrawal response at each of the four larval stages and in young adults suggesting there is no critical period to reverse the effects of isolation on the tap withdrawal response.

**Reversing the Effects of Isolation on Initial Tap Withdrawal Response**

![Graph showing reversal of withdrawal response at each stage](image)

**Figure 3.1.** Critical Periods for Initial Tap Withdrawal Response. Brief mechanical stimulation introduced at the beginning of any stage of development and then tested at 4 days of age (adult) can reverse the effects of isolation. This data suggests there is no critical period to reverse the effects of mechanosensory isolation on the behavioral response to tap so that it can be rescued at any point in development with enough experience.
Fisher's PLSD Comparisons

<table>
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<th>L3</th>
<th>L4</th>
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<td>.04*</td>
<td>.03*</td>
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</table>

Table 3.0 Behavioural Response to Tap. P values using Fisher’s PLSD are reported for each of the 5 experiments. The p values reported compare C (colony) and I (isolate) groups, C (colony) and SI (stimulated isolates), and I (isolate) and SI (stimulated isolate) groups. SC colony groups are not reported because this was a control group and there was no difference detected between the control colony and the stimulated colony group for any experiment (* indicates significance, p<.05)

3.2 EXPERIMENT #2

*Brief mechanosensory stimulation rescues the effects of isolation on post-synaptic glutamate receptor marker GLR-1::GFP at each larval stage suggesting there is no critical period.*

In previous research studies Rose et al. (2003; 2005) investigated the role of glr-1 in the decreased initial response to tap in isolated worms. The responses of a putative null strain of glr-1 (n2461), as well as a rescue strain (kp537), were compared to the response of wild type worms. glr-1 mutant worms reared in isolation responded with the same magnitude of the initial tap withdrawal response compared to colony reared glr-1 mutant worms (Appendix, Figure 4.2). Based on these results, it appears as though the decreased response to tap seen in isolated worms is mediated by the glr-1 type glutamate receptors. Using GFP tagged GLR-1 constructs, Rose et al. (2005) found that worms reared in
isolation expressed significantly less post-synaptic marker GLR-1::GFP compared to colony reared worms; however, brief stimulation during the middle of larval development (L3) rescues the decreased expression of glutamate receptors in isolated worms. This data suggested that there were fewer glr-1 receptors that were expressed on the interneurons of isolate reared worms compared to colony reared worms. By placing animals in an isolated condition, it may have been placed then into a situation in which their deprived nervous system remained in a “waiting state” until mechanosensory stimulation was received. This waiting state may only last for a period of time during development during a window of opportunity where mechanosensory stimulation is needed for the continuance for development; or, it may be that the animal require mechanosensory experience to reverse the effects of isolation however it does not matter at which point in development they experience it. The fact the remarkably little stimulation was required to reverse the effects of isolation on GLR-1::GFP suggests that this system is in some way predisposed to response to experience.

To investigate whether there is a critical period to reverse the effects of isolation on the expression of post synaptic GLR-1::GFP expression, colony, isolate and stimulated isolate groups of the mutant strain GLR-1::GFP were prepared. Stimulated isolated reared animals received brief mechanical stimulation at the start of a specified larval stage (L1-L4, or YAD) of development (using the drop box technique) and later imaged using confocal microscopy at four days of age.

Worms given brief mechanosensory stimulation at the start of L1 and then imaged at four days of age showed that stimulated isolates (n=9) expressed significantly more GLR-1::GFP compared to isolates (n=9) and were no different from colony reared worms
(n=9); L1 experiment, F (2, 25)=6.4, p<0.01. Introducing stimulation at the start of L2 also reversed the effects of isolation as stimulated isolates (n=10) expressed significantly more GLR-1::GFP than isolate (n=10) and were no different from colony reared worms (n=8); L2 experiment, F (2, 25)= 17.8, p<0.0001. Introducing brief stimulation at the start of L3 rescues the effects of isolation as stimulated isolates (n=9) expressed significantly more GLF-1::GFP than isolates raised (n=7) and were no different from colony raised (n=7); F (2, 20)=12.3, p<0.0003). Introducing brief stimulation at the start of L4 also reverses the effects of isolation since stimulated isolates (n=6) expressed significantly more GLR-1::GFP compared to isolate raised (n=7) and were no different from colony raised (n=7); F(2, 17)=23.9, p<0.0001). Finally, introducing stimulation at the start of YAD also reversed the effects of isolation on GLR-1::GFP as stimulated isolates (n=9) expressed significantly more GLR-1::GFP compared to isolate raised (n=9) and were no different from colony raised (n=10); F(2, 25)=16.9, p<0.0001.

In all, I found that brief mechanical stimulation during development rescued the effects of isolation on post synaptic GLR-1::GFP expression at L1, L2, & L3, L4 and YAD suggesting there is no critical period for this measure as was the case for reversing the effects of isolation on the behavioural response to tap.
Reversing the Effects of Isolation on GLR-1::GFP

Figure 3.2 Critical period for reversing the effects of isolation on post synaptic marker GLR-1::GFP. Brief mechanosensory stimulation introduced at any stage of development can rescue the detrimental effects of isolation on post synaptic glutamate receptor marker GLR-1::GFP. This data suggests there is no critical period for reversing the effects mechanosensory deprivation on glutamate receptors (*indicates significance p≤0.05).
Fisher’s PLSD Comparisons

<table>
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<tr>
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Table 3.1 Critical period for post synaptic marker GLR-1::GFP. P values using Fisher’s PLSD are reported for each of the 5 experiments. The p values reported compare C (colony) and I (isolate) groups, C (colony) and SI (stimulated isolates), and I (isolate) and SI (stimulated isolate) groups. SC colony groups are not reported because this was a control group and there was no difference detected between the control colony and the stimulated colony group for any experiment (* indicates significance, p<.05).

CONFOCAL IMAGING USING GLR-1::GFP

Figure 3.2b Changes in glutamate receptors using post synaptic marker GLR-1::GFP. The effects of isolation on GLR-1::GFP expression is shown in the ventral nerve cord of the worm. Representative confocal images of a segment of the ventral nerve of a colony (i), isolate (ii) and stimulated isolate (iii) worm expressing GLR-1::GFP. White areas are clusters of GLR-1::GFP expressed in the ventral nerve cord. These are probably on or in the processes of interneuron of the tap withdrawal response. Isolate (ii) reared worms showed significantly less GLR-1::GFP expression per image than did colony (i) reared and stimulated isolate (iii) reared worms.
3.3. EXPERIMENT #3A

Brief mechanosensory stimulation rescues pre-synaptic vesicular marker \( \text{mec-7}\::\text{SNB}\::\text{GFP} \) only if stimulation is delivered at L1; however, greater amounts of stimulation altered levels at later stages suggesting there is a sensitive period for this effect early in development.

To determine whether mechanosensory stimulation had any effect on the pre-synaptic marker of vesicles I used the construct \( \text{mec-7}\::\text{SNB}\::\text{GFP} \) which marks synaptobrevin (a VAMP that regulates neurotransmitter release) specifically in touch sensory neurons. Rose et al. (2005) demonstrated that worms reared in isolated expressed significantly less pre-synaptic SNB-1::GFP compared to worms reared in a colony. This result indicated that the amount of synaptobrevin and, by implication, the number of synaptic vesicles in the mechanosensory neuron terminals was determined by the rearing condition and by inference, the amount of activity the circuit experienced across development.

Furthermore, Rose et al. (2005) concluded that brief mechanosensory stimulation introduced during the middle of larval development (L3) did not alter levels of the pre-synaptic vesicle marker suggesting stimulation did not alter synaptobrevin expression in the sensory neurons. In addition, studies of long-term memory of habituation in adult worms also failed to find alterations in synaptobrevin with levels of stimulation that were enough to alter post-synaptic marker GLR-1::GFP (Rose et al., 2003). As a result, both Rose et al., 2003 and Rose et al., 2005, showed that \( \text{mec-7}\::\text{SNB}\::\text{GFP} \) required higher levels of stimulation over a greater period of time compared to what was needed to reverse the effects of isolation on GLR-1::GFP. To test this hypothesis, I first tested to see if brief mechanosensory stimulation (30 box drops at a 10s ISI) earlier or later in
development (larval stages L1, L2, L4 and YAD) than compared to L3 (because Rose et al. 2005 only tried reversing these effects at L3) could produce any changes in SNB-1::GFP expression.

Results seemed promising as stimulated isolates (n=6), worms that received brief mechanosensory stimulation at the start of LI, expressed significantly greater SNB-1::GFP compared to isolate reared (n=8) but were no different than colony reared (n=8); F(2, 19)=8.3, p≤0.003. Thus, brief stimulation introduced at the start of larval stage L1 was sufficient to reverse the effects of isolation on SNB-1::GFP expression. In contrast, introducing brief mechanosensory stimulation at the start of L2, showed that stimulated isolates (n=13) expressed the same amount of SNB-1::GFP compared to isolate reared (n=14) and expressed significantly less SNB-1::GFP compared to colony reared worms (n=20); F(2, 44)=5.0, p≤0.01. In support of Rose et al. (2005) I replicated the effects seen at L3 as introducing brief stimulation at the start of L3 did not reverse the effects of isolation on SNB-1::GFP as stimulated isolates (n=10) expressed significantly less SNB-1::GFP compared to colony reared worms (n=6) and were no different from isolate reared worms (n=7); F(2, 20)=7.5, p≤0.004. The same was the case when stimulation was introduced at the start of L4 as stimulated isolates (n=7) expressed significantly less SNB-1::GFP compared to colony reared (n=8) and were no different compared to isolated reared (n=5); F(2,17)=5.4, p≤0.02; and when stimulation was introduced at the start of YAD, stimulated isolates (n=10) still expressed significantly less SNB-1::GFP compared to colony reared (n=8) but were no different than control isolate reared (n=10); F(2, 25)=6.2, p≤0.006.
In all, these results suggest that brief mechanical stimulation only during L1 but not L2, L3, L4 nor YAD rescued SNB-1::GFP expression suggesting two possibilities: 1) there is a critical and or sensitive period for the expression of pre-synaptic vesicles early in development; 2) or that there may be a threshold for the amount of stimulation required to alter levels of SNB-1::GFP that increases with age.

**Reversing the Effects of Isolation on Pre-Synaptic Vesicle Marker** $\gamma_{nec-7}::SNB-1::GFP$ with Brief Stimulation

**Figure 3.3** Critical periods for reversing the effects of isolation on pre synaptic vesicular marker $\gamma_{nec-7}::SNB-1::GFP$. Brief mechanosensory stimulation introduced at the start of larval stage L1 reversed the effects of isolation; however, this same amount of stimulation does not alter the expression of SNB-1::GFP in the touch sensory neurons suggesting there is a critical period during L1 to reverse the effects of isolation on pre synaptic vesicles when introducing only a brief amount of stimulation (30 stimuli at a 10s ISI) (*indicates significance p<0.05).
Table 3.2 Critical period for pre synaptic marker \( \rho \text{mec-7SNB-1::GFP}. \) P values using Fisher’s PLSD are reported for each of the 5 experiments. The p values reported compare C (colony) and I (isolate) groups, C (colony) and SI (stimulated isolates), and I (isolate) and SI (stimulated isolate) groups. SC colony groups are not reported because this was a control group and there was no difference detected between the control colony and the stimulated colony group for any experiment (* indicates significance, p≤.05).

Table 3.2 Critical period for pre synaptic marker \( \rho \text{mec-7SNB-1::GFP}. \) P values using Fisher’s PLSD are reported for each of the 5 experiments. The p values reported compare C (colony) and I (isolate) groups, C (colony) and SI (stimulated isolates), and I (isolate) and SI (stimulated isolate) groups. SC colony groups are not reported because this was a control group and there was no difference detected between the control colony and the stimulated colony group for any experiment (* indicates significance, p≤.05).

**Table 3.2** Critical period for pre synaptic marker \( \rho \text{mec-7SNB-1::GFP}. \) P values using Fisher’s PLSD are reported for each of the 5 experiments. The p values reported compare C (colony) and I (isolate) groups, C (colony) and SI (stimulated isolates), and I (isolate) and SI (stimulated isolate) groups. SC colony groups are not reported because this was a control group and there was no difference detected between the control colony and the stimulated colony group for any experiment (* indicates significance, p≤.05).

**CONFOCAL IMAGING USING \( \rho \text{mec-7::SNB-1::GFP} \)**

(i) Colony

(ii) Isolate

(iii) Stimulated Isolate (at L1)

Figure 3.3b Changes in vesicles concentration using pre-synaptic vesicle marker \( \rho \text{mec-7::SNB-1::GFP}. \) Effects of isolation on \( \rho \text{mec-7::SNB-1::GFP} \) expression in the ventral nerve cord. A representative confocal image of a segment of the ventral nerve cord of a colony (i), isolate (ii) and an isolate stimulated at L1 (iii) expressing \( \rho \text{mec-7::SNB-1::GFP} \) is shown. White areas are GFP expressed in sensory terminals of the tail sensory neurons (PLML and PLMR). Isolate raised worms showed significantly less \( \rho \text{mec-7::SNB-1::GFP} \) in the ventral nerve cord compared to colony and stimulated isolates at L1.
3.3.1 Experiment #3B

Greater amounts of simulation at later stages of development can alter levels of pre-synaptic vesicle marker $\textit{mec-7::SNB-1::GFP}$

To test whether greater amounts of mechanosensory stimulation would produce changes in the levels of pre-synaptic SNB-1::GFP expression, 200 box drops (33.3 minutes) were introduced at the start of L2. Since there was still no difference in pre-synaptic SNB-1::GFP expression, stimulation was increased to 400 box drops (66.6 minutes). Preliminary studies have shown that 400 box drops reversed the effects of isolation on pre-synaptic vesicle marker SNB-1::GFP if introduced at either the start of L2 or L3. Worms reared in isolation (n=10) and who received 400 box drops at the start of L2 expressed significantly greater levels of $\textit{mec-7::SNB-1::GFP}$ compared to control worms reared in isolation (n=10) for the entire duration of development and were no different from control colony reared worms (n=10); $F(2, 24)=3.0$, $p \leq 0.08$. Similar findings showed that if 400 box drops were introduced at the start of L3, stimulated isolates (n=9) expressed significantly greater levels of $\textit{mec-7::SNB-1::GFP}$ compared to control worms reared in isolation (n=11) and were no different from control colony reared worms (n=10); $F(2, 27)=7.9$, $p \leq 0.002$ (Figure 3.4; Table 4). These experiments show that as worms develop greater amounts of stimulation were required to reverse the effects of isolation on the levels of pre-synaptic SNB-1::GFP expression.
Reversing the effects of isolation on pre-synaptic vesicle marker $\text{mec-7}::\text{SNB-1}::\text{GFP}$ with greater amounts of stimulation

Figure 3.4 Reversing the effects of isolation on SNB-1::GFP with greater amounts of stimulation at later stages of development. A) Introducing 400 box drops at the start of L2 or L3 (B) reversed the effects of isolation on pre-synaptic marker SNB-1::GFP. It may take even greater amounts of stimulation to alter levels at L4 and YAD.

Fisher’s PLSD Comparisons

<table>
<thead>
<tr>
<th>Larval age Comparisons</th>
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<td>C, SI</td>
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<td>.32</td>
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<td>I, SI</td>
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</table>

Table 3.3 Fisher’s PLDS comparisons for reversing the effects of isolation on SNB-1::GFP with greater amounts of stimulation at later stages of development. P values from Fisher’s PLSD comparison are represented in table. The levels of total SNB-1::GFP area are compared between C (colony) and I (isolate), C and SI (stimulated isolates), and I and SI groups (*indicates significance, p<0.05).
3.4 EXPERIMENT #4A

Brief mechanosensory stimulation using the box drop technique did not reverse the effects of mechanosensory deprivation on worm length.

Rose et al. (2005) also showed that worms reared in isolation grew to be significant shorter in worm length compared to worms raised in a colony. In the behavioural experiments above, we showed that brief mechanical stimulation at each of the larval stages rescued the initial tap withdrawal response in stimulated isolate worms, but did not rescue the effects of isolation on size. I hypothesized that a greater amount of stimulation may be required to produce changes in body size. To investigate whether there is a critical period and/or there is a critical amount of stimulation required to reverse the effects of isolation on body size, several experiments were run.

Using the box drop technique, 100 box drops (16.6 minutes of stimulation) were administered to the stimulated isolate experimental group at the start of larval stage L2. After 100 box drops during larval development (L2), stimulated isolates (n=16) were still significantly shorter than colony reared worms (n=18) and were no different in size compared to isolated reared worms (n=17); F(3, 64)=15.0, p<0.0001. Following the hypothesis that greater amounts of stimulation would be required to reverse the effects of isolation on worm length, 400 box drops (66.6 minutes of stimulation) were introduced at the start of L2. However, 400 box drops yielded similar findings since stimulated isolates (n=19) were still significantly shorter in worm length compared to colony reared worms (n=19), but were no different in body length than isolate reared worms (n=18); F(3, 71)=6.4, p<0.0007.

This data suggested that neither 5 minutes (30 box drops), 17 minutes (100 box drops), nor more than an hour (400 box drops) of mechanosensory experience introduced
early in development (larval stage L2) had any effects on reversing the effects of mechanosensory deprivation on worm length. Therefore, I altered the design of the experiment to incorporate higher levels of stimulation over longer periods of time during development. These results are outlined in the next experiment.

**Worm Length Measured After 100 Box Drops at L2**

![Bar chart showing mean worm length measured after 100 box drops at L2.]

**Figure 3.5 Worm length: 100 box drops at L2.** Worm lengths of control colony and isolate reared worms were measured at 4 days of age. Isolates that were given 100 box drops at a 10s ISI starting at the beginning of L2 were still significantly shorter in worm length compared to colony reared worms suggesting that 100 box drops was not sufficient to reverse the effects of isolation on worm length (*indicates significance p<0.05).

**Fisher’s PLSD Comparisons**

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<td>C, SI</td>
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**Table 3.4 Worm Length: 100 Box drops at L2.** P values using Fisher’s PLSD are reported for the experiment. The p values reported compare C (colony) and I (isolate) groups, C and SI (stimulated isolate groups), and I and SI. Stimulated isolate worms were given 100 box drops at a 10s ISI starting at the beginning of larval stage L2. This amount of stimulation did not effect worm length as stimulated isolates are no different in length compared to isolates reared worms (*indicates significance p<0.05).
Figure 3.6. Worm length: 400 box drops at L2. Worm lengths of control colony and isolate reared worms were measured at 4 days of age. Isolates that were given 400 box drops at a 10s ISI starting at the beginning of L2 were still significantly shorter in worm length compared to colony reared worms suggesting that 400 box drops was not sufficient to reverse the effects of isolation on worm length (*indicates significance p≤0.05).

**Fisher’s PLSD Comparisons**

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Table 3.5. Worm length: 400 box drops at L2. P values are reported for Fisher’s PLSD. P values represent comparisons between C (colony) and I (isolate) groups, C and SI (stimulated isolate) groups, and I and SI. Stimulated isolate worms were given 400 box drops at a 10s ISI starting at the beginning of larval stage L2. This amount of stimulation did not effect worm length as stimulated isolates are no different in length compared to isolates reared worms (*indicates significance p≤0.05).
3.4.1 EXPERIMENT #4B

*Transferring isolates to a colony condition early in larval development allows the isolated worms to experience greater amounts of mechanosensory experience over a longer time period which rescued the effects of isolation on worm length.*

Since brief amounts of mechanosensory stimulation were not sufficient to reverse the effects of mechanosensory deprivation on worm length, the transfer protocol (Figure 1.2) was used where the worms received greater amounts of stimulation from conspecifics over longer periods of time during larval development. In the transfer protocol worms are transferred from one rearing condition to the other at different stages of development. The first experiment using the transfer protocol involved raising worms in isolation and then transferring them to a colony condition (where they received greater amounts of stimulation over a greater period of time).

Experiments were conducted for the isolate to colony transfer at each age (L1, L2, L3, L4 and YAD) which included three experimental groups of worms: Control colony (raised in a colony for entire life), control isolate (raised in isolation for entire life) and a transferred isolate (worms raised in isolation until a specific age and then all of the worms were transferred to a single colony plate) condition which were compared and measured at four days of age. Results from experiments where isolates were transferred to colony conditions show that worm length is rescued only when worms are transferred to a colony condition early in larval development (L1, L2, and L3) but not later.

Control isolate worms (n=14) were significantly shorter than both control colony (n=13) and isolates transferred to a colony in L1 (n=21); F(2, 45)=6.3, p≤0.004. Control isolate worms (34) were significantly shorter than both control colony (n=54) and isolates transferred to a colony at L2 (n=37); F(2, 131)=3.1, p≤0.05. In addition, control isolate
worms (n=61) were significantly shorter than both control colony (n=68) and isolates transferred to a colony at L3 (n=66); F(2, 192)=11.8, p<0.0001. Since worms reared in isolation and then transferred to a colony condition at L1, L2 and L3 were all significantly greater in body size than worms reared in isolation it suggests that the mechanosensory stimulation received from conspecifics after the transfer was sufficient to reverse the effects of isolation early in development. However, worm length was not reversed in worms reared in isolation that were transferred to a colony condition after larval stage L4; transferred isolates (n=34) at L3 were significantly shorter than control colony worms (n=34) and were no different in worm length compared to control isolate reared worms (n=34); F(2, 99)=11.9, p<0.0001. There was no difference in body size between isolate worms and worms transferred to a colony at L4 (p<0.63); moreover there was no difference in worm length between worms reared in isolation (n=16) and worms transferred from isolation to a colony (n=18) at YAD as they were both significantly shorter than control colony reared worms (n=18); F(2, 49)=15.0, p<0.0001. In L4 and YAD experiments, both isolate worms and transferred isolates were significantly shorter in body length compared to worms reared in a colony suggesting that there is a critical periods to reverse the effects of isolation for worm length that ends by the end of larval stage L3.
Figure 3.7 Isolate to colony transfer. Worms are transferred from individual isolate plates to a single colony plate. This graph is a combination of 5 separate experiments. In each experiment, isolate worms were transferred to a colony plate at specific larval stage (i.e. experiment 1: isolates transferred to a colony at L1) and left in a colony until 4 days of age when they were tested for worm length. The effects of isolation are rescued if isolate worms are transferred to a colony condition at L1, L2 or L3; however, if isolates are transferred to a colony at L4 or YAD, the effects of isolation are not reversed (*indicates significance p<0.05).
### Fisher’s PLSD Comparisons

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**Table 3.6 Isolate to colony transfer.** P values for Fisher’s PLSD are reported for each comparison in each of the 5 experiments conducted. 3 comparisons were made: C (colony) and I (isolates), C and TI (transferred isolates) and I and TI (*indicates significance p<0.05).

#### 3.4.2 EXPERIMENT #4C

*Transferring colony reared worms to isolation at later stage of development did not have any effect on worm length; transferring at early stages of development resulted in shorter body lengths compared to colony worms that were not transferred.*

To compliment the isolate to colony transfer experiment, I repeated the same experiment only this time worms began in colonies and were transferred to isolation at different stages of development. This experiment investigates whether isolation would effect growth even after the worms had mechanosensory experience early in development.

Interestingly, worms reared in a colony condition and transferred to isolation (n=26) in L1 (so most of their development occurs in isolation) were significantly shorter in worm length than control colony worms (n=23) and no different in size compared to control isolate worms (n=25); F(2, 71)=2.9, p≤0.06. This was the same case for worms
reared in a colony and transferred to isolation during L2 since worms reared in colony (n=18) were significantly longer than worms reared in isolation (n=17) and worms transferred to a colony in L2 (n=23); F(2, 55)=5.0, p≤0.01.

An interesting observation occurred when worms were reared in isolation up until L3 and then transferred to and individual colony. There was a significant difference in body length between all three groups: control colony reared worms (n=29) were significantly longer than isolate (n=25) and transferred colony worms (n=46); but control isolate worms were also significantly shorter in worm length compared to colony worms transferred to isolation at L3; F(2, 97)=14.8, p≤0.0001. These results suggest that L3 is an intermediate stage for reversing the effects of mechanosensory stimulation on worm length. Lastly, worms reared in colony and transferred to isolation (n=17) at L4 were significantly larger than control isolates (n=10) and the same size as control colony reared worms (n=20); F(2, 44)=8.4, p≤0.0008. Similar finding was shown when worms were reared in a colony and transferred to isolation (n=17) at the start of YAD as they were significantly larger than isolated raised worms (n=13) and the same size as control colony reared worms (n=17); F(2, 44)=13.5, p≤0.0001. This data supports the observation made in the isolate to colony transfer experiment that there is critical period to reverse the effects of isolation on body size that ends sometime during L3.
**Colonies to Isolates Transfer**

**Figure 3.8 Colony to isolate transfer.** Worms are transferred from a colony condition to individual isolate plates at specific stages of larval development. This graph is a combination of 5 separate experiments. For each experiment worms were transferred from a colony to isolation at a specific larval age (i.e. experiment 1: colony worms transferred to individual isolate plates) and then measure at 4 days of age (*indicates significance p<0.05).

**Fisher's PLSD Comparisons**

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<td>.0008*</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

**Table 3.7. Colony to isolate transfer.** P values for Fisher’s PLSD are reported for each comparison for each of the 5 experiments. The 3 comparisons made were C (colony) versus I (isolates), C and TC (transferred colony worms), and I and TC (*indicates significance p<0.05).
CHAPTER IV
DISCUSSION

4.0 SUMMARY

The primary goal of the experiments conducted in this thesis was to determine whether the effects of mechanosensory deprivation on the behavioural response to tap, body growth and the mechanosensory neuron to interneuron synapse could be reversed with sufficient mechanosensory experience during development. The second goal was to determine whether there were critical or sensitive periods during development or if critical amounts of stimulation were required for reversing these effects of isolation on development. These experiments take advantage of *C. elegans*, a simple model system that has a short developmental timeline which is convenient for conducting developmental experiments especially those investigating critical periods. In addition, this model provided a unique opportunity to analyze the effects of mechanosensory deprivation at the level of the synapse using GFP transgenic mutants.

A summary of the results of the experiments reported here can be seen in table 4.0. In experiments 1 and 2, introducing brief mechanical stimulation at any time during development reversed the effects of isolation on the initial tap withdrawal response and on *glr-1* glutamate receptor expression, suggesting there is no critical period to reverse the effects of isolation during development for these two measures. In experiment 3, low levels of stimulation early in development (at the beginning of larval stage L1), but not later, rescued pre-synaptic vesicular marker *p mec-7::SNB-1::GFP* vesicle expression suggesting there is a period during which brief mechanosensory stimulation can reverse
the effects of isolation very easily. Preliminary results have demonstrated that greater amounts of mechanosensory stimulation (400 box drops at a 10s ISI) delivered at the start of L2 or L3 can reverse the effects of mechanosensory deprivation on pre-synaptic vesicle expression. Finally, in experiment 4, larger amounts of mechanosensory stimulation, using the transfer protocol, showed that rearing worms in isolation and transferring them to a colony at the start of L1, L2 or L3 (and not L4 or YAD) rescued the effects of isolation on body growth suggesting there is a critical period to reverse the effects of isolation on worm length that ends during L3. In a complimentary study, worms reared in a colony and then transferred to individual isolate plates at the start of L1, L2 or L3 were still shorter than colony reared worms when tested at 4 days of age supporting the hypothesis of a critical period to reverse the effects of mechanosensory deprivation on worm length that ends during L3.

<table>
<thead>
<tr>
<th></th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>YAD</th>
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<tbody>
<tr>
<td>Behavioral response</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Post synaptic receptors</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Pre synaptic vesicles</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Worm length</td>
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<td>↔</td>
<td>↔</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

✓ = 30 box drops ✓✓✓ = 400 box drops ↔ = transfer protocol

**Table 4.0. Summary Table of Results.** Brief mechanosensory stimulation (30 box drops) reversed the effects of isolation on the initial response to tap and GLR-1::GFP post synaptic receptor expression at any stage of development. Brief stimulation reversed the effects of isolation on SNB-1::GFP pre synaptic vesicle marker expression only if delivered in L1; greater amounts of stimulation (400 box drops) reversed SNB-1::GFP at later stages. The effects of isolation on worm length were reversed using the transfer protocol (isolate to colony and colony to isolate) when transferred at L1, L2, or L3.
4.1 RELATIONSHIP BETWEEN BEHAVIOURAL PATTERNS & EXPRESSION OF \textit{glr-1} GLUTAMATE RECEPTORS

There was complete concordance between my experiments to rescue the response to tap and experiments to rescue post synaptic \textit{glr-1} receptor marker GLR-1::GFP expression. This suggests that the behavior and glutamate receptors are tightly linked to each other. Indeed, there is abundant evidence that supports the hypothesis that the level of \textit{glr-1} glutamate receptor expression corresponds to the magnitude of the behavioural response to tap in the worm. Using the transgenic strain of worms expressing chimeric receptors made up of GLR-1 receptors tagged with green fluorescent protein (GLR-1::GFP), other studies (Rose \textit{et al.}, 2003; Rose \textit{et al.}, 2005; Ebrahimi and Rankin, submitted) have also shown a similar relationship between behavioural response and glutamate receptor expression in \textit{C. elegans}. Earlier studies that provided a foundation for the experiments conducted in this thesis showed that the effect of isolation on the behavioural response to tap in the colony-isolate paradigm is \textit{glr-1} dependent as there was no difference in their initial response to tap between control colony and isolate worms using the \textit{glr-1(n2461)} mutant (Rose \textit{et al.}, 2005). These results suggest that in \textit{C. elegans} morphological changes at the neural level, specifically changes in glutamate neurotransmission, may underlie the observed changes at the behavioural level. Worms reared in isolation expressed significantly less GLR-1::GFP along the ventral nerve cord of the worm (Rose \textit{et al.}, 2005) than worms reared in colonies. Introducing brief mechanosensory stimulation at any time during development reverses the effects of isolation on GLR-1::GFP since the stimulated isolate worms consistently expressed significantly more GLR-1::GFP than isolate reared worms. Similarly, parallels between
behavioural response and glutamate receptor expression can also be found in studies investigating long term memory in *C. elegans*. Using the same type of imaging and GFP construct, Rose *et al.* (2003) found that long-term memory produces a re-organization of GLR-1 in the ventral nerve cord of the worm. GFP constructs were used to visualize glr-1 expression in the interneurons of the mechanosensory circuit of trained and untrained worms (control) 24 hours after long term memory training. The training experienced by the trained group of worms resulted in a decrease in the amount of GLR-1::GFP expressed at synapse 24 hours after training compared to control worms.

These are very interesting results. There is a strong positive correlation between the behavioural rescue of the effects of isolation and the expression of post-synaptic glr-1 receptors and a strong positive correlation between the effects of long term memory in trained worms and the expression of post-synaptic glr-1 receptors. Both experimental paradigms show behavioural changes with changes in GLR-1::GFP expression following a period of mechanosensory experience. Interestingly, in neither experiment did we find the same trend between the effects of mechanosensory experience on the response to tap and pre-synaptic synaptobrevin. This led us to believe that although the post-synaptic glr-1 receptors are rescued and the animal seems to behave normally, there may be other deficits due to low numbers of vesicles in the mechanosensory neurons.

4.2 RELATIONSHIP BETWEEN THE AMOUNT OF STIMULATION & SNB-1::GFP EXPRESSION

In contrast to the relationship between behavior and glutamate receptors, I found that there was a different relationship between the amount of mechanosensory stimulation
experienced by the worm and the total area of pre-synaptic vesicle marker synaptobrevin (SNB-1::GFP) expressed in the sensory neurons. Worms reared in isolation that were deprived of mechanosensory stimulation express significantly less SNB-1::GFP compared to worms reared in a colony condition (Rose et al., 2005). Experiments conducted in this thesis demonstrate brief mechanosensory stimulation (30 box drops at a 10s ISI) can reverse the effects of isolation on SNB-1::GFP only if the stimulation is introduced early in development (at the start of L1). To reverse the effects of isolation at a later stage of developments greater amounts of stimulation are required to alter levels of SNB-1::GFP as preliminary results show that 400 box drops can reverse the effects of isolation on SNB-1::GFP if it is introduced at the start of L2 or L3. I hypothesize that it will take even greater amounts of mechanosensory experience to alter the effects of isolation on SNB-1::GFP in L4 or YAD. Ongoing experiments are investigating this hypothesis.

It is interesting that worms reared in isolation require greater amounts of mechanosensory stimulation to alter the levels of SNB-1::GFP at later stages in development. This suggests that \( \text{p}_m\text{ec-7::SNB-1::GFP} \) is maximally sensitive to stimulation immediately after hatching and that they become less sensitive as they get older. The cause of this change is currently unknown.

4.3 RELATIONSHIP BETWEEN GLR-1::GFP & SNB-1::GFP

In these experiments we visualized the effects of early deprivation on genes expressed in the nervous system in the living worm by using green fluorescent protein (GFP) tagged genes. Two (GFP) gene fusion constructs were used to observe changes in
morphology at the synapse between the sensory neurons and the command interneurons (a synapse critical for plasticity of the tap response in adult worms; Rose et al., 2003). These constructs allowed visualization of a subunit of a non-NMDA-type glutamate receptor (glr-1) in the command interneurons (GLR-1::GFP; Rongo & Kaplan, 1999) and synaptobrevin, a protein associated with synaptic vesicles, in the touch/tap mechanosensory neurons (mec-7::SNB-1::GFP; Nonet, 1999). I observed significantly less expression of both GFP constructs in the ventral nerve cords of worms reared in isolation, deprived of the mechanosensory stimulation from conspecifics (Rose et al., 2005). These results support the hypothesis that synaptic vesicles in mechanosensory neurons and glr-1 glutamate receptors are determined by the amount of activity the circuit experiences across development. Brief mechanical stimulation during all stages of larval development rescued the expression of the glr-1 glutamate receptors, but not expression of the synapse vesicles.

One surprising result was the different aspects of the synapse require varying levels of mechanosensory experience for normal development. One might expect that here would be a strong relationship between expression levels of pre- and post-synaptic genes, however this was not what I saw, nor was it what was seen in studies of adult plasticity. In all cases GLR-1::GFP appeared to have a lower threshold for change than did SNB-1::GFP. Thus, an interesting conclusion from this thesis is that development of different systems may occur at varying rates so that rescue of one element of a system does not necessarily correlate with other elements. This can be seen in the observations that activity dependent changes in the behavioural response to tap and glutamate receptor expression levels did correlate, while activity dependent changes in post-synaptic
glutamate receptor levels did not always correlate with alterations in pre-synaptic vesicle marker distribution.

There is now mounting evidence that in mammals' plasticity is mediated by the regulation of glutamate receptors (reviewed in Malino and Malenka, 2002) with changes in levels of expression of glutamate receptors leading to changes in synaptic strength. The experiments in this thesis have now extended this by showing effects of rearing condition at the level of the synapse in *C. elegans* by demonstrating alteration in glutamate receptor expression in intact animals in response to increases and decreases in experience across development. The analysis of both pre- and post-synaptic elements of identified neurons and the behavior they mediate in living animals has not been possible in any other system studied to date. These experiments are the first to demonstrate that behavioural experience can alter gene expression in the nervous system leading to changes in behaviour and in development in *C. elegans*.

Rose *et al.* (2005) hypothesized that unless both pre- and post-synaptic markers were rescued then the behaviour would not be fully rescued to the levels of colony worms. One way they have looked at this was to look at the responses to repeated taps to see if the animal habituates to tap in the same way as colony worm. To do this they looked at the habituation curves of colony, isolate and stimulated isolates and saw that the stimulated isolates habituated much more rapidly than the colony worms (Appendix, Figure 4.4). This supported the hypothesis that unless both pre- and postsynaptic markers are rescued, mechanosensory behaviour is not fully rescued. To test this hypothesis further, using the stimulus protocols that rescue the synaptobrevin expression we need to show that the behaviour is fully rescued in these animals. An important aspect of “fully
rescued” will be to test whether these worms can show the normal plasticity (short and long-term habituation) that colony worms show. This is a key study because we cannot stop at analyzing the rescued initial response to tap and assume the effects of isolation have been overcome. This would be a faulty conclusion and would lead to developing treatments that only partially reverse the effects of early deprivation. Furthermore, it would be interesting to test examine patterns of GLR-1::GFP and SNB-1::GFP in a mec-4 background to test whether the lack of sensory neurons to transducer mechanosensory stimulation affects expression of these genes in the same way that isolation does.

4.4 THE EFFECTS OF MECHANOSENSORY DEPRIVATION ON BODY GROWTH

Rose et al. (2005) showed that worms raised in isolation were smaller in worm length and width compared to age matched worms raised in groups. Since Rose et al., (2005) found that worms that developed under isolate conditions had significantly fewer eggs per worm than colony reared worms at 70 hours of age, the size difference between colony and isolate raised worms may be a result of different rates of development in the two conditions. This suggests that isolate raised worms are either defective in egg laying or they are slower in their rate of development.

The experiments in this thesis demonstrated that worms reared in isolation that do not experience any mechanosensory stimulation before the start of larval stage L4, will remain significantly shorter in worm length compared to colony reared worms. The evidence for this comes from experiment 3B where worms reared in isolation and then transferred to a colony either at the start of L1, L2 or at L3 rescued the effects of isolation
on worm length. However, if worms were transferred to a colony condition at the start of L4 or YAD, they remained significantly shorter in length compared to the control colony reared worms. If worm length cannot be reversed if stimulation is introduced at the start of L4, but can be reversed if stimulation is introduced at the start of L3, this suggests that somewhere within larval stage L3 (during which there is a sudden increase in cell proliferation) there exists a critical period to reverse the effects of mechanosensory deprivation on worm length. This hypothesis was further supported in the complimentary study where worms reared in a colony condition were transferred to individual isolate plates at the start of larval stage L1, L2 and L3 remained significantly shorter in length compared to control colony reared worms since most of their developmental period was spent in isolation. On the other hand, if worms were reared in a colony condition and then transferred to individual isolate plates at the start of L4 or YAD, isolation did not affect worm length as they were significantly greater than control isolated worms.

Since Rose et al., (2005) concluded that there is a different genetic pathway for the effects of isolation on the initial tap withdrawal response and on worm length, the greater amounts of mechanosensory experience that were required to alter worm length after isolation must have been working by a different mechanism from that which was activated to reverse the effects of isolation on behaviour.

In the transfer protocol experiments, the independent variable manipulated was the amount of mechanosensory stimulation worms received from conspecifics; however, mechanosensory stimulation may not have been the only variable accounting for the differences in size between colony and isolate reared worms. There are 3 ways to test whether chemosensory cues played a role in the effects of isolation on worm length.
Firstly, by using a double mec-osm (mechanosensory-chemosensory) mutant, I could test whether different sensory systems are additive in their effect on size. Secondly, I could use the osm-6 mutants (mutants that do not detect chemosensation) in the transfer experiment (i.e. isolate to colony transfer) to see if varying amounts of chemosensory stimulation has any effect on size. Lastly, I could insert an active channel such as a rhodopsin-gated channel or a constitutively active channel in the touch cells, to see whether the effects on worm length are primarily influenced by mechanosensation.

4.5 CRITICAL VS. SENSITIVE PERIODS

An important factor in understanding how deprivation affects an organism and how stimulation can be used to reverse some of the effects of early deprivation is whether or not there is a specific, finite time period during which stimulation can exert its effects on the system. This notion of a fixed time during development when some aspect of the organism is susceptible to alteration by experience is called a critical period. Konrad Lorenz carried out some of the first experiments on a behaviour that appears to show critical periods, imprinting in chicks (Lorenz, 1957). He manipulated the age at which newly hatched ducklings were exposed to moving parental substitutes and determined that there was a critical age for imprinting (involving the chick following the individual or object, and then when mature courting the individual or object first seen). Lorenz believed that the onset and offset of this period was determined by intrinsic processes of development under the control of specific genes of the organism. Thus, within a species there would be very little variation in the timing of the critical period, but there could be variation in different species. In studies of social development in monkeys, Harlow and
Harlow (1965) showed that social deprivation in the first 3 months of a monkey's life had many very serious long-term developmental consequences, while deprivation after 6 months of age resulted in relatively mild developmental problems. Traditionally, studies of critical periods during development focused on specific time periods during which the nervous system is more plastic; however, as studies continued, researchers demonstrated that these "fixed" critical periods could sometimes be altered by some aspect of experience; for example many critical periods for visual phenomenon could be extended by rearing animals in darkness. If appropriate neural activation is absent, then the neural circuit remains in a waiting state until the stimulation is received (Hensch, 2004). This is not true of all behaviors that have critical periods; if human language is not learned before 12 years of age, the development of normal language abilities is not possible (Lenneberg, 1964). Thus, the characteristics of critical periods can differ across behaviors within an organism and between different species.

In the studies conducted in this thesis, I did not find any evidence for a critical period for reversing the effects of isolation on either the response to tap or GLR-1::GFP distribution. Rather this appears to be a situation in which the deprived nervous system remains in a waiting state until mechanosensory stimulation is received. The fact that remarkably little stimulation was required to reverse the effects of isolation on these systems suggests that they are, in some way, predisposed to respond to experience. We have not yet tested whether increasing early experience might increase the amplitude of either the behavioral response or GLR-1::GFP expression. It would be interesting to investigate what the outcomes would be for worms raised with 2, 6, 10, 40 or 50 conspecifics to determine whether the behaviour and the glutamate receptors show an
on/off stepwise response (insufficient stimulation versus sufficient) or whether the behaviour and glutamate receptor expression will show a dose response effect with higher levels of stimulation leading to larger responses.

I showed that although the effect of isolation on the initial response to tap and on the expression of the glr-1 receptors on the interneurons was rescued by brief stimulation (30 stimuli) introduced at any stage of development, the vesicular marker of the pre-synaptic terminal (SNB-1::GFP) was only rescued if brief stimulation was introduced very early on in development (at the start of L1). During L1, it takes very little stimulation to reverse to effects of isolation on the pre-synaptic vesicular marker SNB-1::GFP; however, as worms get older they require much more stimulation to reverse the effects of isolation on SNB-1::GFP. Since greater amounts of mechanosensory stimulation (400 box drops) were required to rescue the effects of isolation on SNB-1::GFP at later stages of development (L2 and L3), we know that it can be reversed with sufficient stimulation. This suggests that perhaps there is a sensitive period early in development to reverse the effects of isolation on SNB-1::GFP with very brief mechanosensory stimulation. Konrad Lornez (1957) defined a sensitive period as the time during the organism's development when it is uniquely ready for specific type of input. Since greater amounts of stimulation can rescue these effects at later stages in development, it is not critical that the animal receive this brief amount of stimulation early on in development because it can still be rescued by greater amounts of stimulation at a later age. In other words, the animal is ready for mechanosensory experience very early on in development but will not be shut-out if it does not receive that experience until a later point in development. In the case of SNB-1::GFP, the only catch is that if the
animal receives the necessary experience at a later stage in development, it will required higher levels of stimulation to exert the same effect.

Although I did not find a critical period for rescuing the effects of isolation on the initial response to tap, or glutamate receptors, and found a sensitive period to rescue the effects of SNB-1::GFP, I did find what appears to be a critical period to reverse the effects of isolation on worm length. This critical period extends from hatching until later in development, within larval stage L3. One possible explanation for this is that colony reared worms do not receive ample amounts of mechanosensory stimulation early in development, because they may not be as mobile as they are when they are older. Nevertheless, since our colony condition consists of only approximately 30 worms on a single plate, perhaps a greater density of worms reared in a colony condition would have produced different results. Investigating the effect of increasing population density on response to tap and worm length would test this hypothesis. In this case, worm raised in isolation could be tested against worms raised in colonies of different population densities (5, 10, 20, 40, or 80 worms per colony).

Two important questions that still need to be addressed are: what is occurring in the animal’s nervous system during these critical periods, and why do we find critical periods at these specific points in development? Research on other organisms has demonstrated that experience during development can also influence the structure of neural circuits. Morphological changes in the synapse of neurons are seen in the mammalian brain following environmental manipulations (Greenough, 1975). During development, there may be a time period when neurons may be particularly sensitive to environmental or experience related input. This sensitive period could be sensitive to
environmental or experience related input. In roughly 2 weeks *C. elegans* go through their entire life cycle from embryo to geriatric, but their nervous system is not fully developed in its adult form until the animal is at least 3 days of age (Kenyon, 1998). Thus approximately 20% of the animal’s life span post-hatching is spent developing the nervous system. During development, the length of *C. elegans* increases fivefold (Jorgensen and Rankin, 1997). Many of the anatomical and functional connection are established during the larval stages (White *et al.*, 1986) while the animal is being bombarded with sensory stimuli from the environment. The bouts of neurogenesis and rewiring that occur while the animal is exposed to sensory stimuli from the environment provide a developmental substrate for environmental influence to act on.

**4.6 LIMITATIONS, FUTURE DIRECTIONS & IMPLICATIONS**

The results presented in this thesis illustrate the effects that mechanosensory deprivation have on the behavior, development and the nervous system of *C. elegans*; however there are a few limitations that should be considered when interpreting these results. The first is that I have only investigated the effects of isolation on the behavioral response to tap, post-synaptic glutamate receptors, pre-synaptic vesicles and body size; isolation must affect various other aspects of the nervous system and these elements probably interact with each other to produce final outcomes. For example, I have only explored the effects that mechanosensory deprivation 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. In addition, I have only investigated the effects of mechanosensory deprivation on a single behaviour, the initial tap withdrawal response;
therefore, unraveling the effects of isolation on these four measures is a stepping stone in understanding how mechanosensory deprivation affects the organism as a whole system.

It will be challenging to generalize these results to mammalian model systems for a few reasons. These findings do not directly correspond to mammalian systems because vertebrates are a much more complex system in that they have a spine, intricate brain, and many more neurons and genes. Nevertheless, "a nervous system is nervous system, is nervous system" so there are definitely similarities between the two systems that can be used as important insight into factors related to reversing the effects of isolation on development of the nervous system.

Large-scale parametric studies are much simpler in C. elegans than in other systems. Studying the effects of isolation using the nematode was advantageous because I was able to focus on the effects of mechanosensory deprivation and rule out the effects of all other senses. These effects of isolation studied in thesis were not related to nutritional needs, or temperature changes, but to a lack of tactile stimulation of the worms from conspecifics on the plate. From this research we have expanded our understanding of the factors that influence revering the effects of mechanosensory deprivation in C. elegans.

There are many exciting future avenues for this work in which further investigation of these effects will allow us to characterize the features of these critical and sensitive periods to understand the ways they are different from the periods of time before or after them. Further directions should focus on determining whether certain aspects of the critical periods are determined by the time that has passed since the egg hatched versus whether certain aspects of the critical/sensitive periods are determined by specific developmental stage that the worm has reached. In addition, we need to begin
genetic analyses of candidate genes that might play a role in altering sensitivity to experience in order to develop further understanding of mechanisms governing critical/sensitive periods in *C. elegans*.

By manipulating the expression levels and patterns of the genes that we identify we will extend our understanding of the mechanisms of the critical periods and develop ways to reverse the effects of early deprivation in other systems. If critical periods are regulated by the developmental stage of the worm, and not by the time since hatching, we can alter the rate of development of worms either by using any of a number of mutations that slow development (i.e. *clk-1*), by increasing the temperature to 25 degree Celsius for faster development, or by decreasing the temperature 17 degrees Celsius for slower development compared to the optimal temperature of 20 degrees Celsius to investigate whether a critical is period due to a temporal factor (i.e. hours since fertilization) or whether it is dependent on a specific developmental event or stage.

One possible explanation for the size difference between the colony and isolate worms is that the colony worms detect other worms and speed up their development in order to compete with older worms for resources. It is possible that worms may be able to measure/detect the number of conspecifics present by the number of collisions they encounter. If they detect there are many other worms on the plate, it may influence their rate of feeding. If isolate worms experience no competition they may not have the need to eat as much as colony worms that eat more since they are competing with others for food. This may be reflected in body size since isolates are much shorter than colony reared worms. This could be tested by measuring the effects of different population densities
from isolates to very high density colonies on worm length, behavioural response to tap and rate of pharyngeal pumping.

Understanding how stimulation of the nervous system affects both the nervous system and the organism as a whole can help promote child development and may help to develop remedies for cases of early deprivation. To do this we need to understand how the nervous system changes with early experience, how the mechanisms work to mediate different levels of stimulation and their effects on the nervous system at different times during development, and how to alter the sensitivity after it has changed. In this thesis I have developed a system in which we can study the role of mechanosensory stimulation alone during development in an organism that does not require maternal care to develop normally. The large amount known about the nervous systems and genetics of *C. elegans* makes it an ideal system in which to explore cellular and molecular mechanisms underlying the effects of stimulation on developing nervous systems. The genetic manipulations that are now routine in studies using *C. elegans* will offer various ways to investigate the role of identified genes in determining why some time periods during development are more or less sensitive to stimulation than others. Future work will hopefully continue this investigation of the effects of early stimulation and will develop an understanding of the features of the critical periods and the genes that regulate them to determine ways to alter them to treat organisms deprived of early stimulation.

My results support the notion that development of different systems follows different rules/time courses. Rescuing one aspect of development will not necessarily reverse the total effects of isolation on the developing organism. Different aspects of development will require varying amounts of stimuli at varying time points in
development, which would all need to be taken into account to fully rescue the effects of deprivation on the organism. With this simple model system we have the possibility of determining the cellular bases of such differences.
FOOTNOTE

The work presented in this thesis will be submitted for publication as: CRITICAL AND SENSITIVE PERIODS FOR REVERSING THE EFFECTS OF MECHANOSENSORY DEPRIVATION ON DEVELOPMENT IN CAENORHABDITIS ELEGANS in Developmental Psychobiology.
REFERENCES


Figure 4.1 The magnitudes of standardized reversal response to tap for the colony and isolate groups for N2 (wild type) and osm-6 worms are shown in Appendix 1. An overall ANOVA comparing the mean tap response magnitude for each group showed a significant difference between conditions (F (3, 76) = 6.8; p ≤ 0.0004). Fisher’s planned comparisons indicated significant differences in response magnitude between isolate and colony raised worms in both N2 (p ≤ 0.0034) and the osm-6 strain (p ≤ 0.0017). Based on these results it is highly unlikely that a lack of chemosensory stimulation plays a role in the decreased response to tap of isolated worms.
Figure 4.2 The magnitudes of standardized reversal response to tap for colony and isolate groups of N2, glr-1 and glr-1 rescue worms (kp537) are shown in appendix 2. An overall ANOVA comparing mean tap response magnitude for each group showed a significant difference between rearing conditions (F (5, 476)=5.01; p<.0002). Fisher’s planned comparison indicated significant differences in response magnitude between isolate and colony reared worms in the N2 strain (p<.0002) and the glr-1 rescue strain (p<.015) and no difference between isolate and colony reared worms in the glr-1 strain (p<0.88). Based on these results, it appears as though decreased response to tap seen in isolated worms is mediated by glr-1 type glutamate receptors.
Figure 4.3 We raised N2, mec-4 and egl-4 worms in isolation and in colonies. At four days of age, we compared initial response to tap and worm length. An overall ANOVA comparing mean tap response magnitude for each group showed a significant difference in response magnitude between rearing conditions (F(5, 99)=19.2; p<.0001). Fisher’s planned comparison indicated significant differences in response magnitude between isolate and colony reared worms (p<.0006) and in the egl-4 strain (p<.0007) and no difference between isolate and colony raised worms in the mec-4 strain (p<.93). An overall ANOVA comparing worm length for each group also showed significant difference between rearing conditions (F(5, 99)=53.5; p<.0001). Fisher’s planned comparisons indicated significant differences in worm length between isolate and colony reared worms in the N2 strain (p<.0003) and no difference between isolate and colony worms in the egl-4 strain (p<.96) or in the mec-4 strain (p<.22). Thus N2 worms show effects of isolation on both response to tap and not on worm size, egl-4 worms show effects of isolation on response to tap and not on worm size, whereas mec-4 that do not respond to tap also do not show effects of isolation on worm size.
**Figure 4.4 Short term habituation.** Worm raised in isolation and stimulated in L3 (30 box drops at a 10s ISI) habituate faster to a tap stimulus compared to worms reared in colonies. This behaviour is consistent with the glutamate vesicular transporter mutant (*eat-4*), which has less neurotransmitter available. This suggests that although glutamate receptors are rescued the decrease in glutamate vesicles still can affect behavior, thus the behavior is not fully rescued.