The roles of CREB, CaMK1 and Ageing in Short- and Long-term Tap Habituation in *Caenorhabditis elegans*

by

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Abstract

Changes in synaptic connections and neural excitability are thought to mechanistically underlie learning and memory. This dissertation further extends our understanding of the processes governing learning and memory through experimental studies of short- and long-term habituation to mechanical (tap) stimuli in the nematode *Caenorhabditis elegans*. I investigated the role of the *C. elegans* CREB homologue, *crh-1*, in response to tap habituation. *crh-1* mutants performed smaller reversals in response to tap than did wild-type worms and did not show long-term habituation; however, short-term habituation was normal. Expressing CRH-1 in a subset of interneurons of the tap withdrawal circuit rescued the long-term habituation defects observed in *crh-1* mutants: demonstrating for the first time that CREB is required for long-term habituation and that the reversal interneurons are the locus of plasticity for long-term tap habituation in *C. elegans.* To test whether CaMK1 functioned in learning *in vivo* I tested if strains of *C. elegans* with mutations in the CaMK1 homologue, *cmk-1,* and its upstream activating kinase, CaMKK, (*ckk-1* in *C. elegan*s) could habituate to tap. *cmk-1* but not *ckk-1* mutants performed larger reversals in response to tap and did not habituate as deeply as wild-type worms. This is the first demonstration that CAMK1 is required for learning. An analysis of 46 worm strains with mutations in genes predicted by the literature and/or bioinformatics to be targets of phosphorylation by CaMK1 identified 4 strains that ranged from partial to full phenocopy of mutations in *cmk-1*, suggesting that they may function in the same pathway as CaMK1 in learning. I also performed a large parametric behavioural study of tap habituation over a range of intensities in ageing worms. As worms age from 72 to 120 hrs post-egg lay response probability habituation increased. These age-related changes were reflected by the worms' decreasing capacity to show behavioural discrimination of stimulus intensity as they aged. Optogenetics experiments suggested that the age-dependent changes occur upstream of depolarization of the

mechanosensory neurons. These findings increase our knowledge of the mechanisms that govern habituation and open new doors for further research in this area.

Preface

I have written all of the chapters in this dissertation. My supervisor Dr. Catharine Rankin kindly provided many suggestions for editorial changes. Editorial changes have also been suggested by collaborators (listed in each chapter). I conducted the majority of the work contained here with the following exceptions:

Chapter 2: L. Tang performed the behavioural experiments for figure 2.2.

Chapter 3: K. Lee performed the behavioural experiments for figures 3.1, 3.2 and 3.3. J. Xu assisted with injections of the constructs used in and S. Nijeboer performed the behavioural experiments for figure 3.5. A. Leong performed the behavioural experiments for figure 3.6.

Chapter 4: E. Ardiel performed the behavioural experiments for figure 4.3. A. Giles assisted with the kinetic energy and laser Doppler vibrometry experiments and helped me perform the behavioural experiments for figure 4.4.

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CHAPTER 1: General Introduction

All animals can modify their behaviour based on past experience (learning) and maintain that modification as a memory of that experience. These biological processes are critical for survival, as all animals must adapt to the changing environment they live in. Habituation is defined as decrease in responding to repeated irrelevant (non-predictive) stimuli (Thompson and Spencer, 1966). It may be the most important form of learning as: 1) has been conserved throughout evolution (it has been observed in all organisms tested, from protozoans to humans; Harris, 1943); 2) it is the first form of learning that is observable during an animals development (Rankin and Carew, 1987); and 3) it functions as a form of selective attention – allowing the organism to ignore irrelevant stimuli in its environment so that it can use its limited attentional resources to attend to the predictive stimuli in the environment that are important for survival and reproduction. Similar to other forms of memory, memory for habituation occurs in multiple phases and is dependent upon the pattern of stimulation (e.g. short-term memory and long-term memory) and is known to decline with age. The goal of the research laid out in this dissertation was to add to our understanding of the mechanisms of each of these processes.

1.1 *Caenorhabditis elegans* as a model to study learning and memory

Understanding how we learn and remember needs to be approached from several levels, that of of behaviour, of organization of the nervous system, of the neuron, and of the gene to gain a holistic understanding of learning and memory. The principles that govern the behavioural changes associated with human learning and memory have been studied since Ebbinghaus (1913). Technological progress in the last century has provided researchers with new techniques such as recording of evoked potentials (electroencephalography recordings) and functional magnetic resonance imaging (fMRI) that for the first time allow real time measurements of

neuronal activity in a living human. Exciting as this is, these measurements are limited in that they only measure populations of neurons. We have yet to develop techniques that allow us to ethically observe the changes in neuronal organization, in individual neurons, and in brain gene expression in living humans. This places a significant limitation on our understanding of the processes that govern human learning and memory. These ethical and technological limitations are being addressed by neuroscientists through the field of comparative neuroscience, in which animal models are used for physiological and cellular analyses. One such animal model, *Caenorhabditis elegans*, easily overcomes these technological limitations, and can be studied at the level of behaviour, neuronal organization, single neuron and the gene.

C. elegans is a one millimeter long, transparent, free living soil nematode that navigates through its environment using taste, smell, touch and temperature. In the 1960's, Sydney Brenner developed *C. elegans* as a novel experimental model organism to study the genetics of cell differentiation and development of the nervous system; a task that becomes increasingly difficult when studying more complex organisms. Brenner thought *C. elegans* was an ideal organism to study because of its short life span, reproductive simplicity and small size. *C. elegans* are fully developed in 3 days; making it easy to study a large number of animals, and possible to perform multi-generational studies. One sex is a self-fertilizing hermaphrodite that produces clonal offspring. This characteristic minimizes genetic background differences between individuals. They eat bacteria, are stored on small Petri plates, and strains do not need to be constantly maintained as they can be frozen at -80° C. Over the past 40 years, an increasing number of scientists have chosen to study all aspects of *C. elegans* biology. This has led to further development of the worm as a model system, and now studying this organism is like studying an animal with an instruction manual. Advantages and resources of *C. elegans* now include: i) a fully sequenced and annotated genome, ii) a cell lineage fate map, iii) a complete anatomical

map (including a neuronal wiring diagram), iv) the *Caenorhabditis Genetics Centre* that distributes mutant and transgenic strains, v) fluorescent genetic molecules (such as Green Fluorescent Protein (GFP) and its variants) that can be manipulated to show protein localization and function; vi) and the development of a large number of simple but robust behavioural assays. These tools and advantages make *C. elegans* an ideal model to study the cellular basis of learning and memory.

Though I stress that *C. elegans* is a successful model for this purpose because of its simple behaviour, neural organization and ease of use in the laboratory, it is also an excellent model because it shares many biological similarities with higher animals. The genome of *C. elegans* consists of 96 893 008 base pairs encoding over 19 000 genes and regulatory regions (*C. elegans* sequencing consortium, 1998). In comparison, the human genome is 30 times larger, made up 2.85 billion base pairs, but it only encodes slightly more genes (the total number being somewhere between 20 000 – 25 000; International human genome sequencing consortium, 2004). It is estimated that about 35% of *C. elegans* genes are closely related to human genes. Like higher vertebrates, *C. elegans* neurons are organized into centers of neuronal integration (although the nervous system in general is less centralized than in higher animals), have both electrical and chemical synapses, use most of the same neurotransmitter systems, and use many of the same molecules for cell signaling (reviewed in Hall et al., 2006). For simple forms of learning, such as habituation and classical conditioning, the behavioural rules are the same regardless of the organisms studied (from *C. elegans* to humans), thus it is likely that these simple forms of learning appeared very early in evolution and have been maintained throughout phylogeny. Thus, we believe that many mechanisms of learning discovered in simple systems will generalize to more complex systems.

As with studying any single model organism, the possibility must be considered that the molecular mechanisms uncovered here in *C. elegans* are limited to this species and not conserved across evolution. Another potential limitation is that when only one form of learning is assayed using one behavioural task (such as habituation to mechanosensory stimuli) it could be that these findings are specific to this specific form of learning. Even if this does turn out to be the case (although I feel it is unlikely due to the plethora of examples of conserved molecular mechanisms from *C. elegans* to other species and from simple models of synaptic plasticity to associative learning paradigms) these findings will still act to enhance our understanding of learning and memory by highlighting the diversity of its mechanisms and properties.

1.2 Short-term mechanosensory habituation in *C. elegans*

C. elegans can learn about a wide variety of types of information in its environment. The first and most extensively studied learning paradigm in *C. elegans* is habituation to a mechanosensory stimulus (tap; Rankin et al., 1990). In this learning paradigm the worm receives a mechanical stimulus, a tap, applied to the side of the Petri plate within which it resides. The tap results in the worm changing from a forward crawling motion to a backwards one for a brief period of time, after which it crawls forward again. This response to tap is termed a reversal, and this behaviour is called the tap withdrawal response. The response magnitude, the distance the worm crawls backward, and the probability of responding to tap by reversing decreases with repeated stimulation (Beck and Rankin, 1993; Sanyal et al., 2004); and this decrease is termed habituation (Figure 2.1). In this assay short-term habituation is induced by administering 30 tap stimuli at a 10 or 60s interstimulus interval (ISI), followed by testing for spontaneous recovery at 30 sec, 5 min, and 10 minutes after delivery of the last tap. Short-term habituation differs depending upon the ISI used. Short ISIs produce deep levels of habituation that show rapid

spontaneous recovery, whereas long interstimulus intervals produce intermediate levels of habituation that show slower spontaneous recovery(Rankin and Broster, 1992). This sensitivity to interstimulus interval is a feature of habituation that is present in all species studied (Askew, 1970; Davis, 1970; Groves and Thompson, 1970; Byrne, 1982). These findings suggest that short-term habituation at different frequencies might be mediated by different mechanisms, thus reflecting different forms of short-term memory. Recent research from our lab has supported this hypothesis by identifying one gene that affects habituation at 10s but not at 60s interstimulus intervals (ISI), and a second gene that shows the opposite pattern (unpublished data). Habituation to tap can be distinguished from sensory adaptation or fatigue by testing for dishabituation. After habituation training, an electrical shock can be administered to the worm through the agar. This causes dishabituation, such that worms will respond to the next tap stimulus as if it were novel (Rankin et al., 1990).

The neural circuit that mediates the tap withdrawal response was identified by laser ablating individual neurons and testing the ablated animals for changes in response to tap. This method was used to determine that the circuit primarily consists of 5 mechanosensory neurons, 8 interneurons, and a pool of motorneurons. Three of the mechanosensory neurons, ALM left and right (L/R), are located in the anterior of the body and respond to head touch (which causes worms to perform a reversal). The remaining mechanosensory neurons, PLML/R, are located in the posterior body and respond to tail touch (which causes the worm to accelerate forward). When a tap is administered to the side of the Petri plate, the worms feel a mechanical vibration transmitted through the agar at both the anterior and posterior regions of their body. This causes

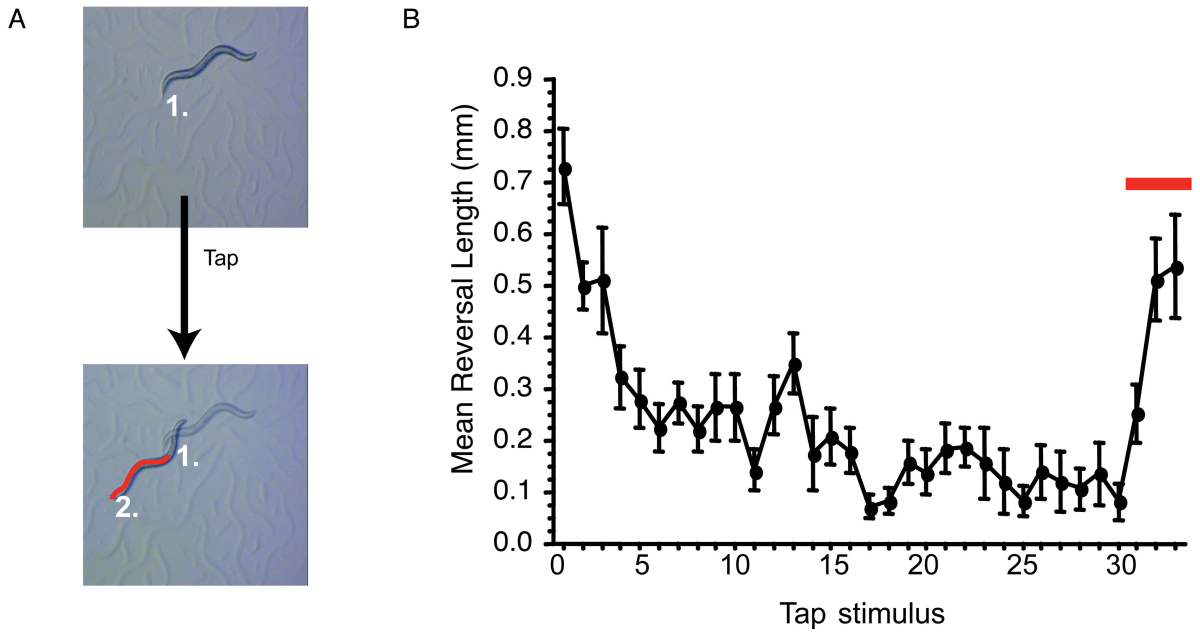

Figure 1.1 Short-term Mechanosensory Habituation in *C. elegans*.

A) Reflexive reversal performed by worms when the Petri plate is tapped (tap withdrawal response). 1- The position of the tail before the tap, 2- the position of the tail after the reversal. The distance the worm reverses is measured (red line). B) Habituation of mean response amplitude (reversal length in millimeters) to 30 tap stimuli delivered at a 10s interstimulus interval. Spontaneous recovery is assayed by tapping worms at 30 s, 5 min and 10 min after the 30th tap (3 responses under the red bar respectively).

"neural competition" within the circuit. Both sets of mechanosensory neurons activate the interneurons (AVAL/R, AVBL/R, AVDL/R, and PVCL/R) but the posterior neurons are hypothesized to do so to a lesser extent, as there are only two of them. This is integrated by the interneurons, signaled to the muscles by the motorneurons and the resultant behaviour is a reversal (Wicks and Rankin, 1995).

Evidence from gene expression studies in the mid 90's led to a hypothesis that glutamate was the neurotransmitter system that mediated the chemical synapses between the mechanosensory neurons and the command interneurons of the tap withdrawal circuit (Hart et al., 1995; Maricq et al., 1995; Dent et al., 1997). Several classes of glutamate receptors are

expressed in the many interneurons of the worm, including on the four pairs of interneurons that are part of the tap withdrawal circuit. Confirmation of the hypothesis that glutamate was a critical neurotransmitter came from the study of the first gene identified to play a role in short-term habituation to tap, EAT-4. This gene encodes the worm homologue of the mammalian glutamate vesicular transporter, VGlut1. EAT-4 loads the neurotransmitter glutamate into synaptic vesicles that are then released when the neuron is depolarized. EAT-4 is expressed in the mechanosensory neurons of the tap withdrawal circuit (Lee et al., 1999), and worms with a loss-of-function mutation in this gene have normal initial responses to tap, but habituate extremely rapidly and cannot be dishabituated by any stimulus (Rankin and Wicks, 2000). This led to the hypothesis that the neurotransmitter between the mechanosensory and interneurons in the tap withdrawal circuit was glutamatergic and suggested that pre-synaptic release of glutamate from the mechanosensory neurons is critical for normal wild-type short-term habituation. Interestingly, normal glutamate transmission appears to be critical for learning in many species across evolution, ranging from habituation, sensitization and classical conditioning in the sea slug, *Aplysia californica* (reviewed in Glanzman, 2007), to the intensively studied cellular models of mammalian learning and memory, long-term potentiation and long-term depression (reviewed in Citri and Malenka, 2008).

Recently, a role for the dopamine neurotransmitter system in habituation to tap has also been demonstrated. In the absence of food, wild-type animals habituated more rapidly than in the presence of food (Kindt et al., 2007); this contextual information about food availability is provided by dopaminergic mechanosensory neurons that sense the presence of bacteria. In the worm, dopamine is synthesized in eight sensory neurons; the two anterior deirid neurons (ADEs), the two posterior deirid neurons (PDEs), and the four CEP neurons that are located in head of the worm (Sulston et al., 1975). A dopamine receptor, DOP-1, is expressed on the

mechanosensory neurons, ALM and PLM, that receive extrasynaptic connections from the dopamine neurons, CEPs and PDEs respectively (Tsalik et al., 2003; Sanyal et al., 2004). Worms with a mutation in the DOP-1 receptor habituated faster than wild-type worms when stimuli were presented at a 10s ISI when habituation was measured by probability of responding to tap. This effect of the dopamine receptor mutation was limited to the rate of habituation, and had no effect on the asymptotic level, and no effect on the spontaneous recovery of the animals. A candidate gene approach to look for mutants that showed the same habituation phenotype as the *dop-1* mutants, followed by double mutant analysis (to determine whether the genes are in the same genetic pathway), revealed genes in the phospholipase-C β (PLCβ) pathway. One of the end-points of the identified PLCβ pathway was the second messenger inositol trisphosphate (IP3). This molecule is a known ligand of IP3-gated calcium channels on the endoplasmic reticulum (ER, an intracellular calcium store). A second PLCβ pathway endpoint, PKC-1, (homologous to mammalian protein kinase C epsilon) can phosphorylate voltage-gated calcium channels. The requirement of these genes suggested a role for calcium signaling in this habituation mechanism. To follow this up, *in vivo* Ca^{2+} imaging of the ALM and PLM mechanosensory neurons revealed that intracellular Ca^{2+} levels increased transiently in these neurons after mechanosensory stimulation, and that the peaks of these transient Ca^{2+} waves decreased with repeated stimulation. When Kindt et al (2007) looked at the effect of mutations in the dopamine-PLCβ pathway, they observed that like the behaviour, peaks of the transient Ca^{2+} waves in ALML/R of mutant worms decreased more rapidly with repeated stimulation than those of wild-type animals.

Behavioural studies of worms with identified neurons laser ablated by Wicks and Rankin (1995) showed that the neural circuit of the tap withdrawal response significantly overlaps with the neural circuits for two other behaviours in *C. elegans;* spontaneous reversals and the thermal avoidance response. These three behaviours share most of the same interneurons and motor

neurons and thus Wicks and Rankin (1997) hypothesized that if the site of neuroplasticity of habituation to the tap withdrawal response was located in the interneurons or motorneurons, habituation training would lead to changes in behaviours that shared those components with the tap withdrawal circuit. On the other hand, if the site of neuroplasticity of the tap withdrawal response was located in the sensory neurons, or their synapses onto the interneurons, they hypothesized that there would be no effect on these other behaviours after habituation training. Their experiments indicated that short-term habituation to tap had no effect on the magnitude or frequency of either spontaneous reversals or the thermal avoidance response. This suggests that the locus of plasticity for short-term habituation is situated presynaptic to the interneurons. The genetic analysis supports and extends the behavioural and neural circuit analysis, and gives us insight onto where in the sensory neurons these changes are happening. The dopamine data points to cellular excitability of the mechanosensory neurons as a locus of neuroplasticity, whereas the EAT-4 data suggests that the synapse could also be a site for plasticity. Thus, it seems that there may be at least two different molecular mechanisms mediating the behavioural plasticity of the tap withdrawal response; a mechanism that modifies the level of mechanosensory cell excitability and a mechanism that modifies synaptic release. It is only through the analysis of this learning at the level of the behaviour, the neural circuit, the individual neuron and the gene that the sites of plasticity were identified to this level of specificity.

1.3 Mechanisms of short-term habituation in other species

The cellular basis of habituation has been studied in the gill withdrawal response in the sea hare (*Aplysia californica*). When a tactile stimulus is applied to the siphon, gill or mantle of *Aplysia* it reacts by reflexively contracting its siphon and gill. This is known as the gill

withdrawal response. Two papers published in the same journal issue first demonstrated that this reflex could habituate (Castellucci et al., 1970; Kupfermann et al., 1970). Repeated tactile stimulation of the siphon or mantle resulted in a gradual decrement of the intracellularly recorded excitatory postsynaptic potential (EPSP) of sensory to motor neuron synapses. Kupfermann and colleagues (1970) were able to develop a semi-intact preparation consisting of the abdominal ganglia (containing the sensory and motor neurons that were connected to the gill, siphon and mantle) and part of the siphon nerve still connected to some of the skin of the siphon. With this semi-intact preparation they observed EPSP decrement at the sensory to motor neuron synapse when they repeatedly stroked the siphon skin. Castellucci et al. (1970) then went on to suggest that the mechanism of this habituation was mediated by synaptic depression of the monosynaptic connection between the sensory and motor neurons as when they electrically stimulated the sensory neurons instead of stroking the siphon skin they were still able to observe decrement of the EPSPs in the motor neurons.

Electrophysiological studies using quantal analysis (a method used to determine what proportion of synaptic change is due to pre- versus post-synaptic changes) suggested that habituation was due to a change in the presynaptic terminal (most likely due to a decrease in the amount of neurotransmitter released from the sensory neurons; Castellucci and Kandel, 1974). Further experimentation showing that habituation could still be observed in the presence of DNQX (an AMPA/kainite-type glutamate receptor inhibitor) helped confirm these findings: blocking post-synaptic AMPA/kainite-type glutamate receptors did not block short-term habituation (Armitage and Siegelbaum, 1998). Further studies attempted to determine the cause of the decline in presynaptic release. Gover and colleagues (2002) developed computational models using known electrophysiological observations of synaptic plasticity in *Aplysia*, and found the model that best fit the previous empirical data was that during low frequency repeated stimulation (such as in habituation) the sensory neurons undergo a step-wise silencing of

neurotransmitter release sites that is independent of exocytosis. They also collected empirical data to test the model and confirmed that this was the most likely mechanism to explain the presynaptic decrease during habituation. The switch of these synapses from active to silent is thought to be initiated by Ca2+ influx, but the mechanism downstream from this initiation is unknown (discussed in Gover and Abrams, 2009).

In mammals an abrupt acoustic, tactile or vestibular stimulus causes a startle response that can be observed as the contraction of skeletal and facial muscles. This reflex has been studied in the rat in the context of habituation. Repeated administration of an acoustic stimulus results in an attenuation of this startle response. The circuit required for this reflex has been defined and consists of multiple sensory inputs coming together from auditory, trigeminal and vestibular sensory pathways synapsing onto a small number of giant neurons in the caudal pontine reticular nucleus. The axons of these giant neurons synapse directly onto motorneurons that innervate the facial and skeletal muscles (reviewed in Koch, 1999). Similar to *Aplysia,* the changes thought to mediate habituation of this reflex occurred presynaptically in the synapse that undergoes synaptic depression (giant neuron to motorneuron synapses; Simons-Weidenmaier et al., 2006). Olfactory habituation in rodents has also been studied (Wilson, 2000, 2009) and pharmacological studies in this model showed that type III metabotropic glutamate receptors mediated this form of habituation (Best et al., 2005). This finding prompted Schmid and colleagues (2010) to test if this was also true for habituation of the acoustic startle response, but they found that *in vivo* application of the mGluRIII antagonist MPPG failed to show any effect on short-term habituation of startle responses suggesting this was not a conserved phenomenon in habituation in rats.

Short-term habituation has been studied in the fruit fly, *Drosophila melanogaster*, in both larvae and adult animals. In larvae, pre-exposure to an odour decreases olfactory chemotaxis (Rodrigues, 1980; Cobb and Domain, 2000; Wuttke and Tompkins, 2000; Boyle and Cobb,

2005) and although it was initially described as adaptation it has subsequently been shown to be habituation (Larkin et al., 2010). Larkin and colleagues (2010) observed that pre-exposing larvae to ethyl acetate for 5 min induced habituation to odour that lasted for up to 20 min that did not require odourant receptor activity, as it could be induced by optically stimulating olfactory sensory neurons expressing the blue-light-activated cation channel channelrhodopsin (ChR2) (ChR2 acts downstream of sensory receptors to activate neurons by directly depolarizing them). They were also able to dishabituate larvae by applying cold shock, further demonstrating that the reduced chemotaxis to ethyl acetate after pre-exposure was indeed habituation and not simply fatigue. Knockdown of GABA-A and NMDA receptors blocked short-term habituation in this paradigm, suggesting they are critical components of this process. They also tested *rutabaga* (homologous to mammalian type 1 adenylate cyclase) mutants on this assay and found that they too were deficient for short-term habituation.

In adult fruit flies habituation has been investigated via two assays, the odour-evoked jump reflex and an assay similar to the one used to test odour habituation in fly larvae. The odour-evoked jump reflex is a behavioural response performed by adult flies in response to air puffs of repulsive odours such as benzaldehyde. Habituation of flies with mutations in *dunce* (homologous to mammalian cAMP phosphodiesterase-4) and *rutabaga* was found to be abnormal using this assay (Asztalos et al., 2007). In the second habituation assay adult flies are exposed to either $C0_2$ or ethyl butyrate (repulsive chemicals) or nothing (naïve group) for 30 minutes and then put into a Y-maze. Flies that were pre-exposed to the repulsive chemicals show a decreased avoidance of them in the Y-maze compared to naïve flies. This habituation can last up to 20 minutes. In the prolonged presence of the repulsive chemical stimulus, olfactory sensory neurons (OSNs) repeatedly activate the projection neurons (PNs). The PNs also receive inhibitory GABAergic inputs from lateral neurons (LNs). The potentiation of this GABAergic synapse from the LNs to the PNs (to inhibit PN depolarization by the OSNs) is thought underlie

habituation via the following mechanism: the GABAergic LNs can also release glutamate that activates NMDA receptors on the PNs when they are coincidently depolarized by the OSNs. When this occurs, such as during habituation, the NMDA receptors send a retrograde signal back to the LNs that potentiates GABAergic signaling from these neurons onto the PNs via cAMP-dependent signaling, resulting in a decrease in activation of the PNs (Das et al., 2011).

Recently, a small molecule screen of 1760 compounds with defined targets was used to identify proteins important for short-term habituation in Zebrafish (*Danio rerio*) (Wolman et al., 2011). Twenty-six compounds were found to alter startle habituation to acoustic stimuli presented at a 20 s ISI. Compounds that inhibited GABA-A and -C receptors, serotonin reuptake, 5-HT2 serotonin receptors, α-1 and α-2 adrenoreceptors, L-type Ca^{2+} channels, D3 dopamine receptors, muscarinic acetylcholine receptors, phosphodiesterase-3, nitric oxide synthase, protein kinase Cs, cyclin-dependent kinases and GSK3β were found to increase habituation, while compounds that inhibited NMDA receptors, L-type Ca^{2+} channels, GABA-A receptors, Cl^- channels, K^+ channels, dopamine receptors, α-adrenergic receptors, Calmodulin, phosphodiesterase-4, cyclin-dependent kinases 1, 2 and 4 as well as myristic acid (known to interact with Src family kinases) were found to decrease habituation. Of these, cyclin-dependent kinases and myristic acid are novel mechanistic components that have not previously been shown to play a role in habituation.

The studies discussed above suggest two possible, but not necessarily mutually exclusive, mechanism for altering the nervous system to produce habituation. In *Aplysia* it has been clearly demonstrated that the behavioural decrement of the gill and siphon withdrawal response during habituation is caused by monosynaptic depression of the synapse from the sensory neurons to the motorneurons. In contrast, recent studies in *Drosophila* have shown that the behavioural decrement of the aversion to repulsive odours in adult flies during habituation is caused by potentiation of an inhibitory synapse that modulates the excitatory circuit that causes the

behaviour. In *C. elegans* it is known that cellular excitability is altered at a 10s ISI during habituation (as measured via calcium imaging) and that mutations in dopamine signaling alter this, but whether this is the only mechanism contributing to short-term habituation at a 10s ISI is still unknown (but currently under investigation; Giles et al., 2011). Almost nothing is known about what cellular mechanisms are responsible for short-term habituation at a 60s ISI in *C. elegans*.

Whether the behavioural decrements caused by habituation are caused by a depression in a monosynaptic connection within the circuit for the behaviour or caused by the potentiation of an inhibitory synapse that modulates the excitatory circuit that causes the behaviour data from studies of short-term habituation in a number of species suggest an important role for presynaptic cAMP and Ca^{2+} signaling in altering neurotransmitter release. Another candidate family of calcium signaling molecules that have not been tested for their role in short-term habituation are the Ca^{2+}/Calmodulin-dependent protein kinases (CaMKs). The catalytic activity of molecules in this signaling cascade are dependent upon increases in intracellular Ca^{2+} concentrations, are highly expressed in the nervous system and have been shown to be important for other forms of learning and memory (reviewed in Wayman et al., 2008a) and thus it is possible that they may also function in habituation.

1.4 Long-term mechanosensory habituation in *C. elegans*

Long-term memory is defined as information stored in the brain that is retrievable over a long period of time (usually > 24 hrs) and is often experimentally induced by the administration of spaced training (rest breaks between training blocks). A difference in memory retention for spaced or massed (no rest breaks during training) training was first suggested in the late 1800's, when Ebbinghaus and others noted that human subjects benefited from spaced practice (Jost, 1897; Thorndike, 1912; Ebbinghaus, 1913). This phenomenon has been observed in rodents

(Goodrick, 1973; Hasegawa et al., 1988; Commins et al., 2003). Recent meta-analyses of the literature on massed versus spaced training in humans have shown quantitatively that several types of learning benefited from spaced practice (Lee and Genovese, 1988; Moss, 1996; Donovan and Radosevich, 1999; Janiszewski et al., 2003; Cepeda et al., 2006).

Despite the fact that worms only live 14-21 days in the lab, they can express long-term memory for habituation (Rankin et al., 1990). Rose et al (2002) developed a procedure to study lasting memory in *C. elegans* that consisted of administering 80 tap stimuli in either a massed or a spaced training protocol. The spaced training protocol involved administering 4 training blocks of 20 taps at a 60s ISI, with a one hour rest period between blocks. In this memory experiment, one group (the trained group) of worms received the spaced training and one group (the untrained group) of worms received only a single tap on the training day. Worms were tested for memory retention 24 hours following training by administering 10 test taps and comparing the response magnitude between the trained and untrained groups. Worms that received spaced training showed smaller reversals to tap during the memory test than did untrained worms that received only a single tap, suggesting the worms had long-term memory for the tap habituation training (Figure 2.2). When the same experiment was performed, but the worms received massed training (80 taps at a 60s ISI) instead of spaced training no difference was observed between the trained and untrained groups 24 hours after training. This indicated that there was no memory retention at this time point (Rose et al., 2002).

Detailed examination of long-term memory for habituation following spaced training in *C. elegans* showed that it shares many characteristics with long-term memories in other animals (including humans). *C. elegans* memory can be long-lasting; initial studies in this organism showed that it lasted from 24 to 72 hrs (Rankin et al., 1990; Ebrahimi and Rankin, 2007). Similar to mammals, and other invertebrates, long-term memory in the worm requires new proteins to be synthesized. This was demonstrated by heat shocking animals after each training

block to halt all ongoing protein synthesis. Worms given this treatment gave reversals during the memory test that were statistically indistinguishable from untrained worms (Beck and Rankin, 1995) . Long-term memory in *C. elegans* was also associated with a decrease in expression levels of a glutamate receptor subunit, GLR-1, (homologous to the mammalian GluR1 glutamate receptor subunit) 24 hours after long-term memory training. Worms with a mutant copy of this gene, *glr-1*, showed wild-type short-term habituation but did not show long-term memory for habituation training (Rose et al., 2003). Trafficking of glutamate receptor subunits is a common theme in studies of plasticity in a variety of organisms. Changes in glutamate receptor subunit expression was correlated with long-term plasticity changes in cellular models of memory, long-term potentiation (LTP) and long-term depression (LTD) in rodents (reviewed in Citri and Malenka, 2008). In LTP, an increase in glutamate receptor subunit expression was observed at the synapses that were stimulated, whereas in LTD, a decrease in the expression of these subunits was seen.

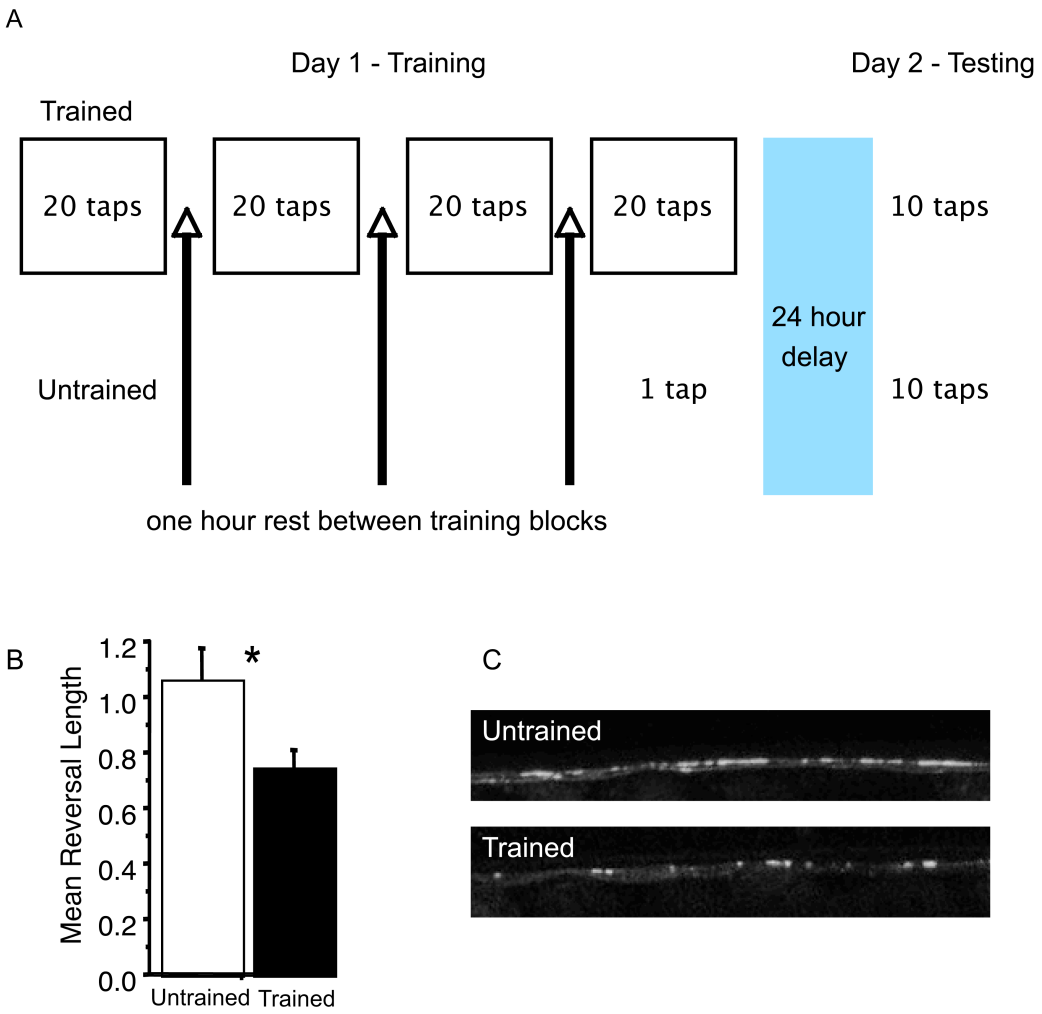

Figure 1.2 Long-term habituation in C. elegans.

A) Long-term habituation training consists of 4 blocks of 20 taps given at a 60s interstimulus interval with a one hour rest period between each training block. Untrained worms receive one tap. The memory test is performed 24 hours later by giving 10 taps to each group. B) Trained worms show smaller responses than untrained worms. C) Fluorescent imaging of GLR-1::GFP fusion protein (expressed in the interneurons of the tap withdrawal circuit) 24 hours after long-term memory training in trained and untrained worms. GLR-1 expression levels are observed to decrease 24 hours after training.

1.5 Mechanisms of long-term habituation in other species

Long-term habituation of the gill and siphon withdrawal reflex in *Aplysia* was first induced by 4 days of spaced training. Each day *Aplysias* were administered 10 weak stimuli to their siphon at a 30 s ISI. This induced a very long lasting memory that was observable weeks later (Carew et al., 1972) and was determined to be mediated by synaptic depression of the sensory-motor neuron synapses that underlies the withdrawal reflex (Carew and Kandel, 1973; Castellucci et al., 1978) and a decrease in the size and number of sensory neuron presynaptic inputs (Bailey and Chen, 1983, 1988). These studies suggested that similar to short-term habituation, a presynaptic molecular mechanism might mediate long-term habituation in *Aplysia.*

To facilitate studying the molecular mechanism mediating these electrophysiological and structural changes in *Aplysia* Ezzeddine and Glanzman (2003) used a reduced preparation. To induce long-lasting habituation in this reduced preparation they administered 4 blocks of 20 bouts of electrical stimulation to the siphon of suprathreshold intensity (just strong enough to reliably elicit gill withdrawal) at a 30 s ISI with a 1-hour rest period between blocks. This protocol was able to induce a long-lasting habituation that was observable at 12 hours after the onset of training. Administration of pharmacological inhibitors of protein synthesis, protein phosphatase 1 and 2A, post-synaptic NMDA receptors and post-synaptic AMPA receptors blocked long-lasting habituation, suggesting these were critical components of long-term habituation in *Aplysia.* A more recent study using the same reduced preparation has also demonstrated a role for RNA synthesis, protein phosphatase 2B/Calcineurin and L-type Ca^{2+} channels in this process (Esdin et al., 2010). These results strongly suggest that a presynaptic mechanism alone is not sufficient to explain long-term habituaiton in *Aplysia*, post-synaptic changes must also be occurring and suggest a conservation of mechanism between long-term habituation and synaptic LTD.

Studies of long-term habituation have also been performed in the crab (*Chasmagnathus granulatus*). In the wild, Crabs are preyed upon by gulls flying overhead and so they respond to a potential danger stimulus, such as a dark object passing overhead, by attempting to run away. In the lab this is measured by keeping crabs in a bowl so that they cannot escape and using an actometer to record their activity in response to a screen being passed overhead. Crabs habituate to the repeated presentation of this stimulus. To induce long-term memory in the crab they are presented with the potential danger stimulus (screen passed overhead four times) 15 times at a 180s ISI. This training will induce a memory that last for up to 5 days. Administering blockers of RNA and protein synthesis inhibited the expression of this training during memory tests (Pedreira et al., 1995; Pedreira et al., 1996; Hermitte et al., 1999). Further studies using pharmacology, and molecular biology have shown that muscarinic acetylcholine receptors (Beron de Astrada and Maldonado, 1999), NMDA receptors (Troncoso and Maldonado, 2002), cAMP signaling (Romano et al., 1996; Locatelli et al., 2002) and the transcription factor Rel/NF-κB (Freudenthal and Romano, 2000) are also important for long-term habituation.

Studies of long-term habituation have also been performed in the rat using habituation of startle to acoustic stimuli. Little is known about the mechanism mediating this form of memory. What is known is that the neural circuit for long-term habituation includes circuitry that was not required for short-term habituation (cerebeller vermis, Leaton and Supple, 1986) and that a vesicular acetylcholine transporter was found to be important for long- but not short-term habituation (Schmid et al., 2011).

In *Drosophila*, long-term habituation has been induced by exposing flies to a repulsive odour, usually benzaldehyde, for 4 days, removing them from the odour overnight and then testing their repulsion to that odour the following day in a T-maze. Their behaviour was compared to that of an untrained group of flies that was exposed to paraffin oil (vehicle) in place of benzaldehyde and other odourants (Devaud et al., 2001). Flies with mutations involved in

cAMP signaling, *dunce* (homologous to mammalian cAMP phosphodiesterase-4), *rutabaga* (homologous to mammalian type 1 adenylate cyclase) and *amnesiac* (homologous to vertebrate pituitary adenylate cyclase-activating peptide, PACAP), did not show long-term habituation to benzaldehyde exposure (Devaud et al., 2003; Keene et al., 2004). Recent studies using this paradigm with $C0_2$ or the odour ethyl butyrate in place of benzaldehyde have also identified miRNA pathway proteins that mediate translation repression (Argonaute 1, Me31B, and Ataxin-2) as being critical for long-term habituation (McCann et al., 2011). Habituation of the CO_2-evoked response has also been shown to be dependent upon changes in Ca^{2+} concentrations in specific regions of the brain but the mechanism of how these changes in Ca^{2+} concentration mediated long-term changes in behaviour is still unknown (Sachse et al., 2007; Suh et al., 2007).

Long-term habituation has also been demonstrated recently in Zebrafish (Wolman et al., 2011). Zebrafish equilibrated to a uniform source of white light respond to a brief (1 s) flash of dark by performing a behaviour known as an O-bend. They habituate to repeated dark flashes and spaced training (4 blocks of 120 dark flashes presented at a 15 s ISI) induced long-term memory observable 24 hours after training. This long-term habituation also required protein synthesis: administering the protein synthesis inhibitor cyclohexamide during training attenuated memory for the training when tested 4 hours later.

Taken together, the findings on the mechanisms of long-term habituation suggests that cAMP and Ca^{2+} signaling cascades, ligand-gated ion channels, gene transcription and protein translation are critical for this process. But how do cAMP and Ca^{2+} signaling mediate gene transcription and protein translation? The transcription factor Rel/NF-κB has been implicated in long-term habituation in the crab but there is another likely but not yet tested candidate: in other forms of long-term memory a transcription factor called the cAMP response element binding (CREB) protein has been shown to be critical for memory (Kaang et al., 1993; Bourtchuladze et al., 1994; Yin et al., 1994; Bernabeu et al., 1997; Guzowski and McGaugh, 1997; Josselyn et al.,

2004). Thus it is possible CREB may function during long-term memory of habituation training as well.

1.6 Ageing and habituation in *C. elegans*

Similar to other biological processes, learning and memory functions are known to decrease during ageing. One study has investigated short-term habituation to tap in *C. elegans* in the context of ageing and found that it too changed with age (Beck and Rankin, 1993). They tested worms that were 4, 7 and 12 days post-egg lay found that as worms aged i) spontaneous and tap-elicited reversal size decreased with age, ii) habituation at a 60s ISI, but not a 10s ISI, increased with age (i.e. worms habituated more deeply), iii) 12 day old worms were slower to recover from habituation training than 4 or 7 day old worms, and iv) no age-related changes in dishabituation were observed. Thus habituation to tap increases as *C. elegans* age but it is not known what causes these age-dependent changes nor is it known if these changes occur across the whole tap withdrawal circuit or if they are caused by age-dependent changes in only a portion of this neural circuit.

1.7 Ageing and habituation in other species

The effects of ageing on habituation have also been studied in the crab and *Aplysia*. Tomsic and Maldonado (1996) found no effect of age on short-term habituation in the crab; but, in contrast Rattan and Peretz (1981) observed that age affected habituation in *Aplysia*. They observed that older *Aplysia* (2 months older than reproductively mature animals) habituated faster and to a deeper level compared to reproductively mature animals and could not dishabituate. They investigated the electrophysiological properties of the L_7 neuron (a neuron critical for dishabituation in mature *Aplysia*) and found that they differed in older animals, specifically the L_7's input resistance was decreased, its time constant was increased and its the

post-synaptic potential size was also decreased. Rattan and Peretz (1981) suggest that the correlative age-dependent changes in L_7's electrophysiological properties may be responsible for the age-dependent changes observed in habituation but causality has yet to be demonstrated.

1.8 Overview of objectives

There remain many unanswered questions in the field of habituation. It is clear that memory of habituation can exist in multiple phases dependent upon training including long-term memory, but it is not known whether the transcription factor CREB, which is a critical component of many other forms of long-term memory, is also a critical component for long-term memory of habituation training. Another unanswered question stems from the role of Ca^{2+} signaling in altering neurotransmitter release in habituation – although some of the downstream components of Ca^{2+} signaling have been identified, no single alteration in these identified components has so far has completely abolished habituation and thus it is likely that other unidentified calcium signaling molecules might also be important for habituation. The catalytic activity of the Ca^{2+}/Calmodulin-dependent protein kinases (CaMKs) are dependent upon increases in intracellular Ca^{2+} concentrations, are highly expressed in the nervous system and have been shown to be important for other forms of learning and memory and thus it is possible that they may also function in habituation. Lastly, ageing is well known to alter cognitive processes, including learning and memory, but there is no consensus in the literature as to when this begins. Studying when and how ageing begins in a simple form of learning, such as habituation, may help elucidate this.

The specific objectives for the research program to further extend our understanding of the processes governing learning and memory documented in this dissertation are:

1) To determine whether the transcription factor CREB plays a role in long-term habituation and to determine the locus in the nervous system where the changes required for long-term habituation occur.
2) To determine whether the Calcium/Calmodulin-dependent protein kinase cascade plays a role in short-term habituation.
3) To explore whether age-related changes in habituation occur during young and middle-aged worms and if so investigate their cause.

CHAPTER 2: CREB is Required in the Interneurons of the Tap Withdrawal Circuit

2.1 Introduction

Changes in synaptic connections and neural excitability are thought to mechanistically underlie learning (Hebb, 1949; Kandel and Spencer, 1968; Martin et al., 2000). In turn, the long-lasting changes at the synaptic and/or neural level that encode long-term memories are thought to be mediated by specific alterations in gene expression. One of the main players known to regulate gene expression changes during the formation of long-term memory is the transcription factor CREB (cAMP response element binding protein). CREB was found to be a critical protein for the formation of long-term memory in invertebrates (Kaang et al., 1993; Yin et al., 1994) and in vertebrates (Bourtchuladze et al., 1994; Bernabeu et al., 1997; Guzowski and McGaugh, 1997; Josselyn et al., 2004). It is known to be an activity-dependent transcription factor, which is activated by phosphorylation of the serine 133 residue (reviewed in Mayr and Montminy, 2001) most commonly by either the cAMP signaling pathway (Gonzalez et al., 1989), MAP kinase pathway (Arthur et al., 2004; Sindreu et al., 2007) or the calcium-calmodulin (Ca^{2+}-CaM)-dependent protein kinase pathway (West et al., 2001).

Although the literature implies that CREB is required for all forms of long-term memories, the role of CREB in long-term memory of habituation training has only been investigated previously in rats (Vianna et al., 2000; Moncada and Viola, 2006). These studies determined that CREB does not play a role in long-term habituation of spatial novelty but functions as a molecular switch between spatial novelty and familiarity. Thus, to date, although CREB is thought to be required for all forms of long-term memory, no one has specifically demonstrated a role for it in long-term memory for habituation.

C. elegans has been a useful tool for investigating the behavioural parametrics of long-term memory of non-localized mechanosensory (tap) habituation training (reviewed in Giles and

Rankin, 2008). The neural circuit mediating the tap withdrawal response was determined using laser ablation studies. It includes 5 sensory neurons (2 pairs of ALM and PLM, along with the single AVM neuron) and 4 pairs of interneurons (AVA, AVD, AVB and PVC) that drive pools of motor neurons (Wicks and Rankin, 1995). Short-term habituation has been hypothesized to occur as a result of changes in the sensory neurons (Wicks and Rankin, 1997), however, to date no one has identified the locus of the site of plasticity for long-term habituation.

Recent studies using this model of long-term memory have begun to elucidate some of the molecular mechanisms involved. Beck and Rankin (1995) determined that this memory relied on protein synthesis, and Rose, Kaun and Rankin (2002) along with Rose, Kaun, Chen and Rankin (2003) demonstrated that this memory was dependent upon the AMPA/kainite-type glutamate receptor subunit, GLR-1. This receptor is expressed in the interneurons of the tap withdrawal response and in 8 other neurons outside of this circuit (Hart et al., 1995; Maricq et al., 1995), suggesting that the locus of plasticity of long-term habituation could be located within the command interneurons (AVA, AVD, AVB and/or PVC). Rose et al. (2003) also discovered that the expression level of GLR-1 tagged with green fluorescent protein (GLR-1::GFP) in the posterior ventral nerve cord of *C. elegans* decreased 24 hours after long-term memory training, and that like the memory itself, this change was also dependent on protein synthesis. The stable change (as long as 48 hours; Rose & Rankin, 2006) in the behaviour of the worms and in the level of GLR-1 after training suggests, that like other long-term memories, long-term memory in *C. elegans* most likely relies on tightly regulated changes in gene expression. CREB is a likely candidate to regulate those changes. The *C. elegans* genome was found to encode a single homologue to the vertebrate CREBs, *crh-1*. The amino acid sequence of this protein is highly conserved in the two domains that are critical for its function; the activation domain (kinase-inducible domain) and the dimerization and DNA binding domain (basic and leucine zipper domain) shared 80 and 95% sequence identity, respectively, with vertebrate CREBs (Kimura et

al., 2002). A recent study demonstrated that *crh-1* was required for an associative form of long-term memory in *C. elegans* (Kauffman, Ashraf, Corces-Zimmerman, Landis & Murphy, 2010), but it is not known if *crh-1* is also required for non-associative long-term memory in *C. elegans*.

I tested the hypothesis that in *C. elegans, crh-1* is required for long-term memory of a non-associative learning task: tap habituation. I found that a *C. elegans* strain carrying a loss-of-function mutant allele of *crh-1* (a deletion of the dimerization and DNA binding domain) was unable to form long-term memory of habituation training. I also found that the rescuing CRH-1 in a subset of the interneurons in the tap withdrawal circuit was sufficient to rescue the long-term memory deficits in these mutants. Here, I provide the first evidence for the requirement of CREB in long-term memory of habituation, and define the locus of plasticity for long-term habituation in this circuit.

2.2 Methods

2.2.1 Strains and maintenance

Worms were cultured on Nematode Growth Medium (NGM) seeded with *Escherichia coli* (OP50) as described previously (Brenner, 1974). N2 Bristol and YT17 *crh-1(tz2) C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). The plasmid pYT41.3.1 (containing *crh-1* rescues driven by the *cmk-1* promoter) was a gift from Y. Kimura (Mitsubishi Kagaku Institute of Life Sciences, Japan). This plasmid was modified for this work by digesting with BamHI and SpHI to remove the *cmk-1* promoter. The *mec-4* promoter (~ 2 kb upstream of ATG; forward primer: CAGACAGCATGCCTATCATTGAGCCGAATATC, reverse primer: CAGAGAGGATCCTTTGAGGCAAGATAAGATC) and the *magi-1* promoter (~ 2 kb upstream of ATG; forward primer: CAGACAGCATGCAGAGCATCCAAGGGATCAA, reverse primer: CAGAGAGGATCCCTTTTTAGTCGTGGGTTCAT) was amplified via PCR using primers

containing SpHI and BamHI restriction digest sites at the 5' and 3' end of the promoter sequences respectively. The PCR products were then ligated into pYT41.3.1 to replace the *cmk-1* promoter using T4 DNA ligase. All plasmids were sequenced to verify identity. Plasmids were injected into young adult *crh-1(tz2)* mutants along with the co-injection marker (plasmid containing *Pmyo-2*::GFP). Stable F2 lines with transmission of *Pmyo-2*::GFP > 50% were selected for. The following strains were created for this work:

VG49 *crh-1(tz2)*; *Pcmk-1*::CRH-1β; *Pmyo-2*::GFP

VG162 *crh-1(tz2)*; *Pmagi-1*::CRH-1β; *Pmyo-2*::GFP

VG166 *crh-1(tz2); Pmec-4::*CRH-1β; *Pmyo-2*::GFP

2.2.2 Behaviour image acquisition and scoring

Worms were visualized using a digital video camera (Panasonic Digital 5100) mounted on a stereomicroscope (Wild M3Z, Wild Leitz Canada). Camera output was fed into a VCR (Panasonic WJ-810) and subsequently into a TV monitor (PM-1271A, NEC). A time-date generator (Panasonic WJ-810) superimposed the time and date on the recorded video. For short-term habituation, stimuli were non-localized mechanical taps that were administered to the side of the Petri plate (containing the worms) via electrical stimulation by a Grass S88 stimulator (Quincy, MA) connected to a mechanical tapper that exerted 1-2 N of force to the side of the Petri plate. For training for intermediate- and long-term habituation the non-localized mechanosensory stimulation was delivered in a different manner. In order to train multiple plates of worms at the same time, plates containing worms were placed in plastic boxes and dropped onto a hard surface from a height of 5 cm. These worms were then tested using the mechanical taps (as described for short-term habituation) 12 (intermediate-term memory) or 24 (long-term memory) hours after training.

VHS videotapes of the testing phases of the short-, intermediate- and long-term habituation assays were scored using stop-frame video analysis. The magnitude of each reversal performed by each worm after the administration of a tap was traced onto acetate sheets. The acetate sheets containing the tracings were then scanned (Epson Perfection 2480 Photo) into an eMac computer (1.25 Ghz Power PC G4). The length of each reversal was measured using NIH image software (version 1.57). Adult worms respond to tap with either a pause, a reversal or a forward acceleration, with the latter occurring about 10% of the time (Chiba and Rankin, 1990). The neural circuit that mediates forward accelerations to tap is different from the circuit that mediates the reflexive reversals and habituates with different kinetics (Wicks and Rankin, 1995; Wicks et al., 1996) and therefore these responses are considered to be qualitatively different from each other. For these reasons, in the current analyses pauses and no response to tap were scored as a reversal distance of "0" and accelerations to tap were scored as missing data points.

2.2.3 Response to tap stimuli and short-term habituation

Ten to fifteen 4-day old worms were transferred using a platinum wire pick from a 96-hour-old synchronous worm colony to an *E.coli* seeded test plate. The plate containing the worms was placed in the tapping apparatus and allowed a 6 minute recovery period before taps were administered. Thirty taps were administered at either a 10s or a 60s interstimulus interval (ISI). The worms' responses were recorded as described above.

2.2.4 Intermediate-term habituation assay and analysis

Fifteen to twenty young adult worms from 72-84-hour-old synchronous worm colonies were transferred to each *E.coli* seeded training plate and placed on a vibration proof shelf. At 96 hours the plates in the training group were placed in a plastic box and dropped repeatedly from a height of 3cm. This method of non-localized mechanosensory stimulation was used to train many

plates of worms at one time. Mechanosensory stimulation administered by tapping the side of the plate (as described for short-term habituation above) or by dropping the plate from a height of several centimeters elicit the same behavioural output (a reversal response) and are sensed by the same sensory receptors (the mechanosensory neurons of the tap withdrawal circuit); animals with mutations in a subunit of the channel that mediates mechanotransduction in these neurons, MEC-4, did not respond to gentle touch or to either non-localized mechanosensory stimuli described above (unpublished observations; Chalfie et al., 1985; Wicks and Rankin, 1995). Using the box-drop method of training, worms received either spaced training (4 blocks of training with an hour rest between each block, during each block the box was dropped at a 60s ISI for 20 drops) or massed training (80 drops at a 60s ISI). Following the last tap of training the test plates in the untrained group received a single box drop. One hour following training the worms were transferred to freshly seeded test plates (15-20 worms per plate). All plates were then placed on a vibration proof shelf for 10 hours. Worms were tested for memory 10-16 hours after administration of the last training tap by placing plates in the tapping apparatus and administering 5 taps at a 60s ISI. The worms' responses to taps were recorded as described above.

2.2.5 Long-term habituation assay and analysis

Fifteen to twenty young adult worms from 72-84-hour-old synchronous worm colonies were transferred to each *E.coli* seeded test plate and placed on a vibration proof shelf. At 96 hours the test plates in the training group were placed in a plastic box and dropped repeatedly from a height of 3cm. Worms received 5 blocks of training with an hour rest between each block (during each block the box was dropped at a 60s ISI for 20 drops). Following the final block of training the test plates in the untrained group received a single box drop. One hour following training the worms were transferred to freshly seeded test plates (15-20 worms per plate). All

plates were placed on a vibration proof shelf for 22 hours. Worms were tested for memory 22-28 hours after administration of the last training tap by placing plates in the tapping apparatus and administering 5 taps at a 60s ISI. The worms' responses to all taps were recorded as described above.

2.2.6 Statistical analysis

All data analyses were conducted using Statview 4.5. Factorial ANOVA's were conducted on all experiments. Post-hoc analyses were comprised of Fisher's post-hoc least significant difference (PLSD) and differences were considered significant when the probability of a Type I error was less than 5%. Single group experiments were analyzed using unpaired two-tailed t-tests. In graphs, responses are expressed as percent initial or untrained response (where appropriate; responses are divided by mean untrained or initial response and multiplied by 100).

2.3 Results

2.3.1 Response to tap stimuli and short-term habituation by *crh-1* mutants

Similar to wild-type animals, untrained adult *C. elegans* with a mutation in *crh-1* responded to tap by reversing. There was no significant difference in the probability of animals that responded to tap between the two strains; 96.4 % of *crh-1* mutants and 100% of wild-type animals responded to the tap stimuli, $t(57) = 1.05$, $p=0.30$ NS. However, the reversal magnitudes of the *crh-1* mutants in response to tap were smaller than those elicited by tap in wild-type animals, $t(57) = 3.25$, $p < 0.002$. The mean (+/- SEM) reversal distance in response to tap for crh-1 mutants and wild-type worms was 0.92 mm (+/- 0.10 mm) and 1.40 mm (+/-0.09 mm), respectively.

Figure 2.1A shows the habituation curves of wild-type and *crh-1* mutant worms tapped at a 10s ISI and figure 2.1B shows the habituation curves of these strains of worms that were tapped

at a 60s ISI. To remove the confound of differing initial response magnitudes to tap between the two strains and compare their rate of habituation I standardized the responses elicited by each tap from each strain to their initial responses to tap. Regardless of ISI tested, *crh-1* mutants habituated at a similar rate and to a similar asymptotic level as wild-type worms; suggesting CRH-1 is not required for short-term habituation. A two-way ANOVA on the responses to tap by wild-type and *crh-1* mutant worms when stimulated at a 10s ISI revealed a significant main effect of stimulus ($F(29,1305)=$, $p<0.0001$), indicating that both strains habituated to the tap stimuli, showed a significant main effect of strain ($F(1,1305)=12.57$, $p=0.0004$), but no significant interaction between stimuli and strain ($F(29,1305)=1.41$, $p=0.07$ NS). Visual inspection of figure 2.1A suggests that the strain difference might be due to only 2 or 3 points; to test this hypothesis I investigated the main effect of strain further using a Fisher's PLSD test no significant difference was observed ($p=0.052$ NS). Although these post hoc analyses determined that there is not effect of strain, the p-value returned is close to significance, thus I investigated this difference further by comparing the response of each strain to each stimuli using an unpaired t-test. These analyses demonstrated that the strain difference results from significant differences between the two strains at only tap number 3 ($p=0.02$) and tap number 22 ($p=0.004$), whereas the t-test analyses revealed no other significant differences between the responses of the two strains to the other 28 tap stimuli. A two-way ANOVA on the responses to tap by wild-type and *crh-1* mutant worms when stimulated at a 60s ISI revealed a significant main effect of stimulus ($F(29,891)=3.97$, $p<0.0001$), indicating that both strains habituated to the tap stimuli, showed no significant main effect of strain ($F(1,891)= 1.82$, $p=0.18$ NS), but did show a significant interaction between stimuli and strain ($F(29,89)=1.525$, $p=0.04$). I further investigated the difference in response to each stimuli by each strain using an unpaired t-test and found that out of the 30 stimuli presented only the responses given to the 4^{th} and 27^{th} tap were significantly different between the two strains of worms ($p=0.031$ and $p=0.0024$, respectively), whereas for

the other 28 stimuli administered no significant difference in reversal size in responses to tap between the two strains was observed. Thus overall I conclude that *crh-1* does not play an important role in short-term habituation.

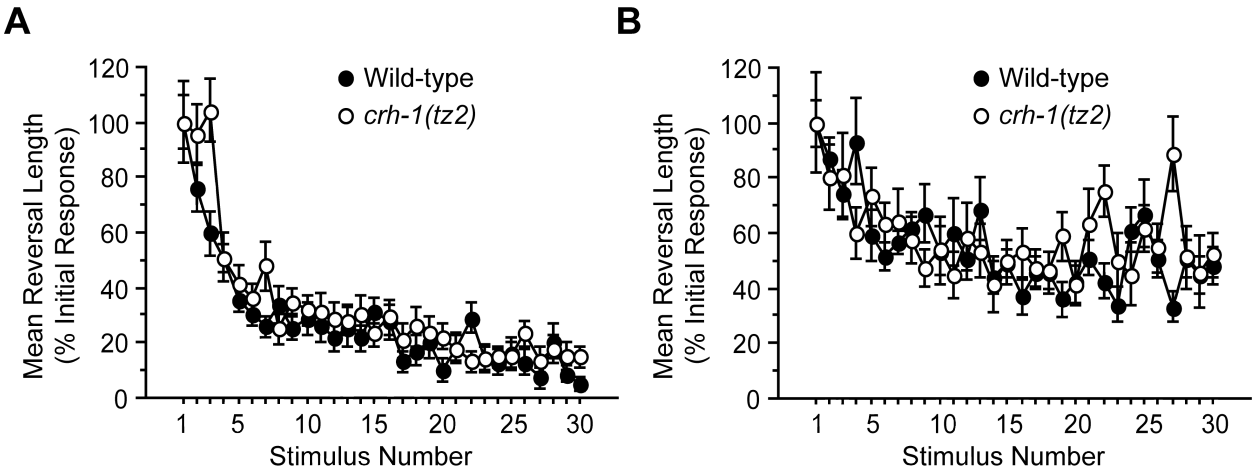

Figure 2.1 Short-term habituation of *crh-1* mutants.

Mean reversal length (+/- SEM) to a series of 30 taps for wild-type and *crh-1* mutants when stimuli were presented at a 10s ISI (A) and a 60s ISI (B). No difference in habituation curves was observed between wild-type and *crh-1* mutants at either ISI. Mean reversal length is represented as a percentage of the mean reversal length in response to the first tap by each strain.

2.3.2 Intermediate-term habituation in *crh-1* mutants

Memory for massed or spaced habituation training 12 hours post training is not dependent on protein synthesis (Rose, 2005) and so I hypothesized that the transcription factor CRH-1 would not be necessary for these types of memory. Figure 2.2A shows the response to tap of untrained and trained worms 12 hours after intermediate-term massed habituation training and figure 2.2B shows the response to tap of untrained and trained worms 12 hours after intermediate-term spaced habituation training from wild-type and *crh-1* mutants. An ANOVA revealed a significant main effect of group in the massed trained protocol ($F(3,659)=104.59$, $p<0.0001$) and in the spaced trained protocol ($F(3,510)=36.18$, $p<0.0001$). My hypothesis was correct; both wild-type and *crh-1* mutants were able to learn and show memory; the worms that received habituation training 12 hours earlier gave smaller reversals in response to tap than did

untrained worms, regardless of the strain or training protocol (wild-type massed, p<0.0001; *crh-1* massed, p<0.0001; wild-type spaced, p<0.0001; *crh-1* spaced, p=0.0007; with Fisher's PLSD method).

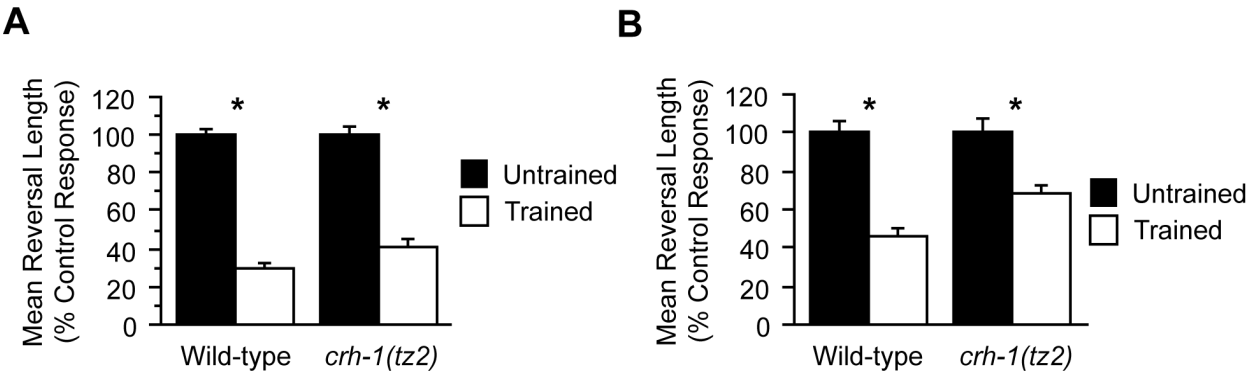

Figure 2.2 Intermediate-term habituation of *crh-1*mutants.

Mean reversal length (+/- SEM) of wild-type and *crh-1* mutants to 5 test taps presented 12 hours after either massed (A) or spaced (B) training. *crh-1* mutants were able to form intermediate-term memories of habituation training regardless of the training protocol. Mean reversal length is represented as a percentage of the mean reversal length in response to tap by the untrained group of each strain. Asterisk (*) represents statistically significant differences greater than p≤0.0007.

2.3.3 The role of *crh-1* in long-term habituation

Figure 2.3 shows the response to tap of untrained and trained worms 24 hours after long-term spaced habituation training from wild-type, *crh-1* mutants, and *crh-1* mutants that were injected with wild-type CRH-1β under control of the *cmk-1* promoter (Pcmk-1::CRH-1β; this promoter drives expression in most neurons in the *C. elegans* nervous system). An ANOVA revealed a significant main effect of group, $F(5,576)=4.23$, $p=0.0009$. As seen in earlier studies, wild-type worms that received habituation training 24 hours earlier gave smaller reversals in response to tap than did the untrained worms ($p=0.02$). In contrast, this was not observed in *crh-1* mutants; in the *crh-1* mutant strain there was no difference observed when I compared the average reversal magnitude in response to tap between the trained and untrained worms ($p=0.28$ NS). When Pcmk-1::CRH-1β was used to express CRH-1 broadly in the nervous system but not in other tissue (Kimura et al, 2002) in *crh-1* mutants, the worms did show memory of the

previous training; the trained worms gave smaller responses to the test taps than did the untrained worms (p=0.01). These data together suggest that CRH-1 expression in neurons is necessary for long-term memory of habituation training.

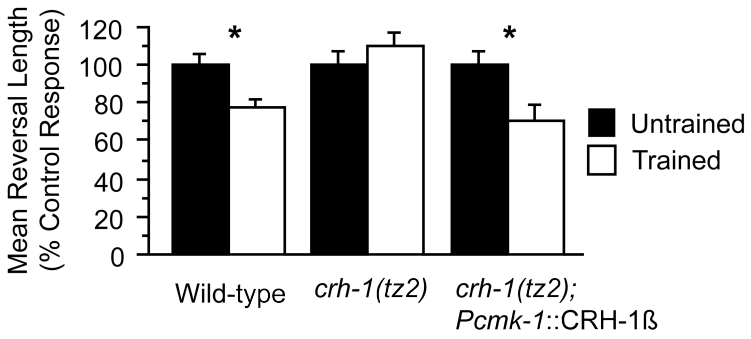

Figure 2.3 Long-term habituation of *crh-1*mutants.

Mean reversal length (+/- SEM) of wild-type, *crh-1* mutants and *crh-1* mutants expressing the cDNA of CRH-1β in the nervous system to 5 test taps presented 24 hours after spaced training. Wild-type worms were able to show memory of habituation training, but no difference between trained and untrained *crh-1* mutants was observed 24 hours after training, suggesting *crh-1* mutants are incapable of forming long-term memories. When CRH-1β is rescued in neurons under control of the *cmk-1* promoter these animals are then able to form memories. Mean reversal length is represented as a percentage of the mean reversal length in response to tap by the untrained group of each strain. Asterisk (*) represents statistically significant differences greater than p≤0.02.

I also investigated subsets of the neurons that make up the tap withdrawal circuit to determine which require wild-type CRH-1 for long-term habituation. I used the *mec-4* promoter to drive expression of CRH-1 in the 6 mechansosensory neurons (ALML and ALMR, AVM, PLML and PLMR, and PVM) and the *magi-1* promoter to drive expression of CRH-1 in the interneurons that mediate reversals, AVA and AVD. The *magi-1* promoter also drove expression in several other neurons (AVE, RIM, RIA SMD, RMD and RMDV; Emtage et al., 2009; Stetak et al., 2009), none of which have been shown to be part of the tap withdrawal circuit.

Figure 2.4 shows the response to tap of untrained and trained worms 24 hours after long-term spaced habituation training from wild-type and *crh-1* mutants that were injected with wild-

type CRH-1β under control of either the *mec-4* or the *magi-1* promoter. An ANOVA revealed a significant main effect of group, F(5,1936)=3.45, p=0.004. Again, wild-type worms that received habituation training 24 hours earlier gave smaller reversals to tap than did the untrained worms (p=0.001). Interestingly, worms with *Pmagi-1* driven CRH-1 and wild-type worms that received habituation training 24 hours earlier gave smaller reversals in response to tap than did the untrained worms (p=0.02). In contrast, in the *Pmec-4* driven CRH-1 rescue worms there was no difference observed when I compared the average reversal magnitude in response to tap between the trained and untrained worms (p=0.7 NS).

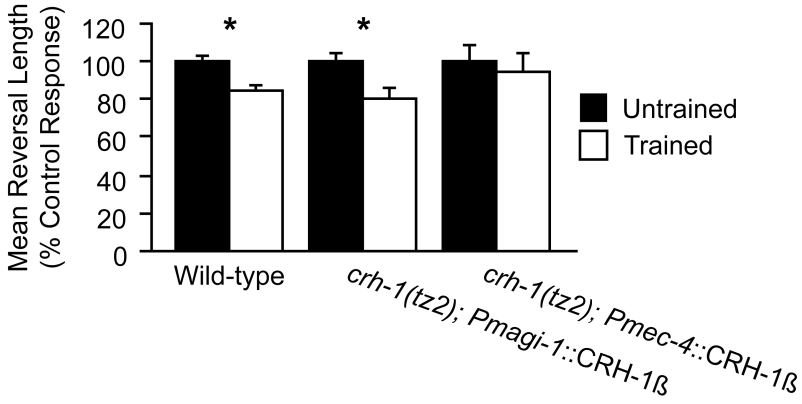

Figure 2.4 Localization of CRH-1 function in long-term habituation.

Mean reversal length (+/- SEM) of wild-type, *crh-1* mutants, *crh-1* mutants expressing the cDNA of CRH-1β in the reversal interneurons of the tap-withdrawal circuit (*crh-1*(tz2); Pmagi-1::CRH-1β) and *crh-1* mutants expressing the cDNA of CRH-1β in the mechanosensory neurons (crh-1(tz2); Pmec-4::CRH-1β) to 5 test taps presented 24 hours after spaced training. Wild-type worms and *crh-1* mutants expressing CRH-1β in the reversal interneurons of the tap-withdrawal circuit were able to show memory of habituation training, but no difference between trained and untrained *crh-1* mutants expressing CRH-1β in the mechanosensory neurons was observed 24 hours after training. Mean reversal length is represented as a percentage of the mean reversal length in response to tap by the untrained group of each strain. Asterisk (*) represents statistically significant differences greater than p≤0.02.

These data show that expression of CRH-1 in the interneurons, but not the sensory neurons, of the tap withdrawal circuit is sufficient for long-term memory of habituation and suggests that these neurons are the site of plasticity for this memory.

2.3.4. Protein kinases and long-term memory

Activation of CREB is known to require phosphorylation of Ser 133 in the kinase inducible domain of the protein. Many protein kinases have been shown to be capable of phosphorylating CREB (reviewed in Johannessen et al., 2004) and thus I tested if various kinase mutants were able to form long-term memory for habituation. I observed that reversal distance during memory testing was dependent on training (figure 2.5, $F(1,2592)=9.371$, $p<0.0001$). Twenty-four hours after spaced training wild-type, *ckk-1(ok1033)* (homologous to mammalian CaMKK) and *pkc-2(ok328)* (homologous to mammalian classical protein kinase C family) mutant worms showed memory of habituation training: the trained worms of these genotypes gave smaller reversals compared to untrained worms of their respective genotypes (wild-type $p<0.0001$, *ckk-1(ok1033)* $p=0.03$, *pkc-2(ok328)* $p=0.002$). Interestingly, two mutant alleles of *cmk-1 (oy21* and *ok287)* (homologous to mammalian CaMK1) and one allele of *pkc-1(ok563)* (homologous to mammalian protein kinase C epsilon and eta, PKCε and PKC**η**) did not form memory for spaced training. In worms of these genotypes responses of trained worms were not significantly different from responses of untrained worms (pNS for all three comparisons). Thus it is possible that either (or both) CMK-1 and PKC-1, but not CKK-1 nor PKC-2, may function to phosphorylate CREB during long-term memory induction in this paradigm.

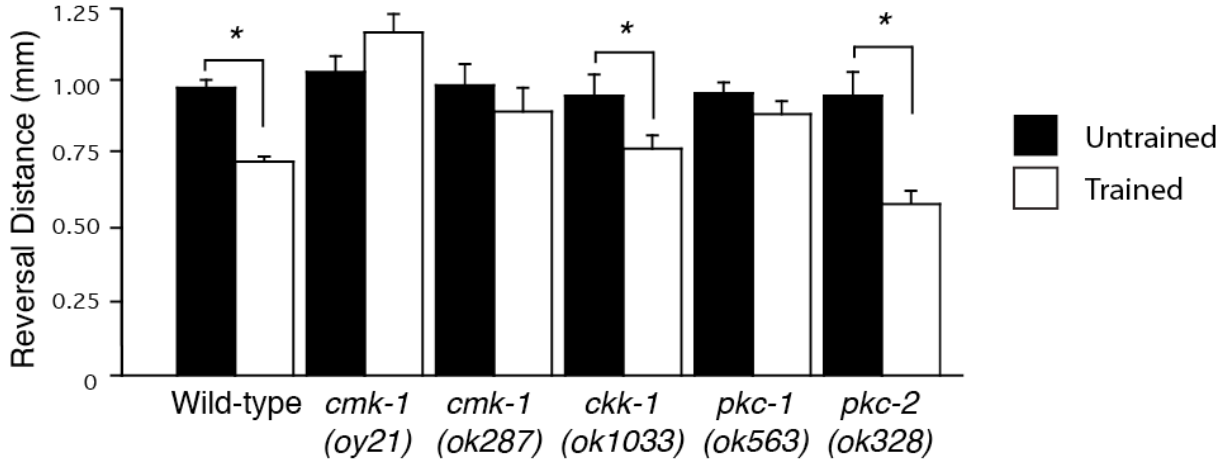

Figure 2.5 Long-term habituation of kinase mutants.

Mean reversal length (+/- SEM) of wild-type and five kinase mutants in response to 5 test taps presented 24 hours after spaced training. Wild-type and *ckk-1 and pkc-2* mutants were able to form long-term memories of habituation training, while *cmk-1* and *pkc-1* mutants were not. Asterisk (*) represents statistically significant differences greater than $p<0.05$.

I also tested a mutant allele of the *C. elegans* homologue of the catalytic subunit of protein kinase A (*kin-1(ok338)*). Because ok338 homozygotes arrest as embryos I tested ok338 heterozygotes. In this experiment, I again found that reversal distance during memory testing was dependent on training ($F(3,81)=17.706$, $p=0.01$). Both wild-type and *kin-1(ok338)* heterozygotes were able to form long-term memory of habituation training; worms that had received spaced training 24 hours earlier responded to taps during training with significantly smaller reversals compared to untrained worms from their respective genotypes (figure 2.6, pNS for both genotypes). It is important to note though that it is possible that one copy of *kin-1* is sufficient for wild-type function, and thus it cannot be ruled out that *kin-1* still might function in long-term memory of habituation. Further experiments using cell specific knockdown of *kin-1* could help elucidate this.

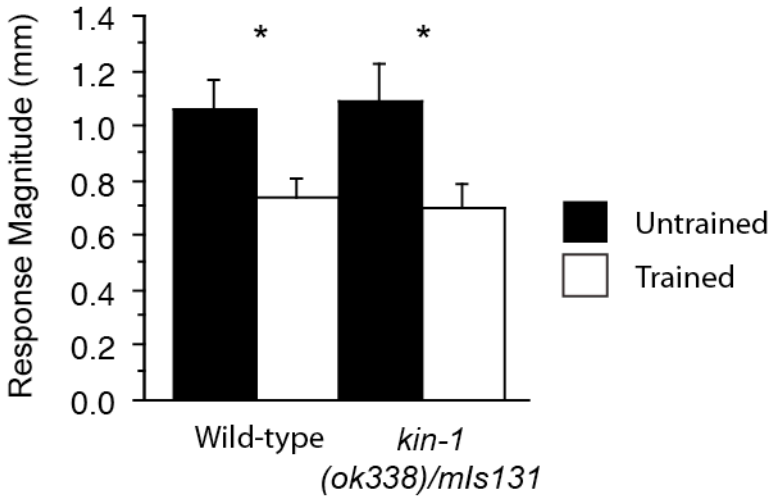

Figure 2.6 Long-term habituation of *kin-1* heterozygotes.

Mean reversal length (+/- SEM) of wild-type and *kin-1* heterozygous mutants in response to 5 test taps presented 24 hours after spaced training. Wild-type and *kin-1* heterozygous mutants were able to form long-term memories of habituation training. Asterisk (*) represents statistically significant differences greater than $p<0.05$.

2.4 Discussion

In this study I investigated the role CRH-1 plays in response to tap and in memory of tap habituation training in *C. elegans* and found that a mutation in *crh-1* that rendered the gene product non-functional blunted the worms response to tap and blocked long-term, but not short- or intermediate-term memory of habituation training. I demonstrated that the loss of the ability of *crh-1* mutants to form long-term memory is indeed due to the deletion in the dimerization and DNA binding domain of the protein and not due to other background mutations in the strain I used by rescuing the memory defects by expressing the beta splice variant of CRH-1 in the nervous system using the broadly expressing *cmk-1* promoter. I also showed that the long-term memory defect observed in the *crh-1* mutants was not due to a floor effect; although *crh-1* mutants make smaller than wild-type initial responses to tap, they were able to habituate and show even smaller reversals than their initial response. This was observed in both short-term habituation and intermediate-term memory for spaced and massed habituation training in this study. To my knowledge, this was the first time CREB has been shown to be critical for the formation of long-term memory for response habituation. I also defined the locus of plasticity for long-term memory of mechanosensory habituation in *C. elegans* by showing that rescuing CRH-1 in a subset of interneurons in the tap withdrawal circuit, AVA and AVD (and possibly RIM), was sufficient to rescue the long-term memory deficits of the *crh-1* mutants.

Previously our lab demonstrated that long-term memory of tap habituation training requires protein synthesis but we were unable to test whether gene transcription was also required. When protein synthesis was blocked using heat shock after training, no memory of the training was observed 24 hours later (Beck and Rankin, 1995). Heat shock was used because it is known to disrupt all ongoing protein synthesis except that of heat shock proteins (the production of which is massively increased; Schlesinger et al., 1982; Lindquist, 1986; Nowak, 1993) and because it

could be delivered in a temporally discrete manner. Previous attempts to block protein synthesis or gene transcription using drugs during spaced habituation training, were not successful because exposure to translation and transcription blocking drugs for the entire long-term memory training period (> 7 hours) made the worms sick and prevented their behaviour from being assayed (previous unpublished results from our lab). My findings here, that a transcription factor was required for long-term memory of tap habituation training, supports the hypothesis that this memory does indeed require *de novo* transcription of new genes. Thus, taken together these data give further support to the notion that this long-term memory is similar to those found in other species, which are dependent on protein synthesis and gene transcription (reviewed in Alberini, 2009).

The locus of plasticity for short-term habituation to tap was shown to be located at the level of the mechanosensory neurons (ALMs, AVM and PLMs) and their synapses onto the command interneurons by Wicks and Rankin (1997); the locus of plasticity for long-term habituation was not addressed in this study. The first pieces of evidence that the locus of plasticity for long-term memory might be different from that of short-term memory came from Rose *et al.* (2003). They demonstrated that GLR-1, expressed in the command interneurons (AVA, AVB, AVD and PVC) and several other pairs of interneurons (AVE, AIB, RMD, RIM, SMD, AVG, PVQ, and URY; Brockie et al., 2001), was required for long-term memory for habituation training. In this study I attempted to confirm that the command interneurons are the site of plasticity for long-term habituation by expressing wild-type CRH-1 in *crh-1* mutants either under control of the *mec-4* promoter or the *magi-1* promoter. Since I did not observe long-term memory in the *Pmec-4*::CRH-1ß worms, I can conclude that the mechanosensory neurons are not the site of plasticity for this long-term memory. Interestingly, I did observe long-term memory in the *crh-1* mutant worms expressing *Pmagi-1*::CRH-1ß, suggesting the site of plasticity for long-term habituation is located within the neurons that express MAGI-1. Because

glr-1 is also required for long-term habituation, the list of potential neurons can be decreased to neurons which express both GLR-1 and MAGI-1, these are: AVA, AVD, RIM, RMD and SMD. Of these, only AVA, AVD and RIM have been shown to play a role in wild-type reversals in response to gentle touch or tap (Chalfie et al., 1985; Wicks and Rankin, 1995; Pirri et al., 2009).

Chalfie et al. (1985) determined that the anterior touch circuit sensory neurons were the ALMs and AVM, while the interneurons were the AVAs and AVDs. Stimulation of the anterior end of the worm by gentle body touch resulted in a reversal. The posterior touch circuit sensory neurons were determined to be the PLMs and the interneurons to be the AVBs and PVCs. Stimulation of the posterior end of the worm by gentle body touch resulted in an acceleration forward. The tap withdrawal circuit was shown to be composed of these two antagonistic circuits: the anterior touch and posterior touch circuits (Wicks and Rankin, 1995). It is hypothesized that when a worm is tapped, the worm senses the vibration through the agar on both the anterior and posterior portions of its body, activating both circuits. It is thought that tap leads primarily to reversals because there are more sensory neurons in the anterior portion of the worm, resulting in greater excitation of the reversal circuit (command interneurons AVA and AVD mediate reversals). The experiments showing that rescuing CRH-1 in AVA and AVD, but not in AVB and PVC, was sufficient to rescue the long-term memory defects observed in *crh-1* mutants suggests that the mechanism for long-term habituation to tap acts via modulating the reversal circuit and not the forward locomotion circuit.

White, Southgate, Thomas and Brenner's (1986) reconstruction of the *C. elegans* nervous system by serial section electron microscopy revealed that the majority of chemical synapses between the interneurons in the touch circuits were from AVD to AVA. AVD received electrical synapses (presumably excitatory) from the anterior touch neurons but had few (a handful) connections to the motor neurons and had to pass the majority of its message to the motor neurons via chemical synapses, which are thought to be glutamatergic, to AVA. AVA, on the

other hand, synapsed heavily on the motor neurons. Thus, I hypothesize that the synapses from AVD to AVA are the site of synaptic plasticity responsible for long-term habituation behaviour. Although this is a prediction that remains to be tested; these data overall suggests that long-term habituation may be caused by synaptic depression between AVD and AVA.

Another interneuron/motorneuron, RIM, has been shown to be activated by AVA and AVD in response to touch and then to inhibit motor neurons and contraction of muscles surrounding the head. RIM was shown to modulate the size of reversals by releasing tyramine (which acts on *lgc*-55; a tyramine-gated chloride channel which is expressed in AVB) to inhibit forward locomotion (Alkema, Hunter-Ensor, Ringstad, & Horvitz, 2005; Pirri, McPherson, Donnelly, Francis & Alkema, 2009). Both GLR-1 and MAGI-1 are known to be expressed in RIM, and thus I cannot completely rule out the possibility that the defects in *glr-1* mutants and the rescuing effects of *Pmagi-1*::CRH-1ß in *crh-1* mutants were, in part, due to their roles during induction of long-term memory in RIM, as well as, or instead of, AVA and AVD. Despite this possibility I hypothesize that the most likely locus of plasticity is still AVA and AVD because of the observation by Rose et al. (2003) that 24 hours after long-term memory training a decrease in GLR-1::GFP was seen in trained, but not untrained worms: of the neurons that express GLR-1 and MAGI-1, only AVA and AVD project into the ventral nerve cord – RIM's projections are restricted to the nerve ring. This is evidence that at a minimum, AVA and AVD are part of the locus of plasticity, but again, a role for RIM in long-term memory by changes in RIM's release of tyramine to inhibit AVB cannot be discounted.

A long-term habituation screen of candidate kinase genes that might function to phosphorylate CREB during long-term memory induction resulted in the identification of 2 potential candidates, CMK-1 and PKC-1. CMK-1 has not been shown previously to play a role in long-term memory in *C. elegans*, nor has its mammalian homologue CaMK1. During development of the nervous system, NMDA receptor-dependent activation of CaMK1γ causes

CREB-regulated transcription of Wnt-2 and microRNA132 indirectly via the MEK/Erk pathway (Wayman et al., 2006; Wayman et al., 2008a), and thus it might be possible for CMK-1 to cause indirect phosphorylation of CREB via a mechanism similar to this during the induction of long-term memory in *C. elegans*.

An alternative possibility is that CMK-1 is functionally equivalent to both CaMK1 and CaMK4. The *C. elegans* genome does not encode a CaMK4 gene, nor does any other invertebrate genome, so perhaps *cmk-1* is similar to the ancestral CaMK from CaMK1 and CaMK4 evolved. One piece of evidence that supports this hypothesis is the subcellular localization of CMK-1. Similar to CaMK1, the *cmk-1* gene contains a nuclear export signal and can be localized to the cyptoplasm (Eto et al., 1999; Satterlee et al., 2004), but interestingly, this gene also contains a nuclear localization signal that is absent in CaMK1, but present in CaMK4. This sequence appears to be functional, as CMK-1 was localized to the nucleus when expressed in COS-7 cells, and deleting it resulted in its exclusion from the nucleus (Eto et al., 1999). Unlike CaMK1, CaMK4 has been implicated in long-term memory, as inhibiting CaMK4 function via dominant negatives or knockouts in mice resulted in animals that perform poorly on tests of long-term spatial and fear memory, deficiencies in forms of LTP and LTD across many regions of the brain, and decreased levels of CREB phosphorylation (Ho et al., 2000b; Wei et al., 2002; Bhattacharyya et al., 2009).

The other gene that might have a role in long-term memory induction is PKC-1. PKCε, homologous to PKC-1, has been shown to phosphorylate CREB in breast cancer cells (Shankar et al., 2010), but to the best of my knowledge, there are no known examples of this in the nervous system. Despite this, other PKC isoforms have been shown to play a role in learning and memory. There is evidence to suggest that PKC in *Aplysia* may function to help degrade CREB1b (a repressor of CREB1a-dependent gene transcription) during long-term facilitation, although this may be occurring indirectly via MSK (Upadhya et al., 2004). In mice, inhibition of

PKCβ caused impaired long-term memory of tone and contextual fear conditioning as well as a decrease in CREB phosphorylation after training compared to control animals (Ahi et al., 2004). Thus it may be possible that PKC-1 could act to phosphorylate CRH-1 during long-term habituation training in *C. elegans.*

The long-term tap habituation experiments on worm strains with mutations in *cmk-1* and *pkc-1* are preliminary evidence that these genes may function in long-term memory, but further experimentation must be done before it can be concluded that they function in this process. First, it is important that genetic rescue or RNAi knockdown experiments be performed to confirm that it is the known mutations in *cmk-1* and *pkc-1* in these worm strains that are responsible for the observed long-term memory phenotypes. Second, cell specific rescue experiments must be done to test if they function in the same cells as CRH-1 during long-term memory induction. And lastly, the level of activated CRH-1 should be assayed in these mutants to determine if loss of function in these kinases results in decreased CRH-1 phosphorylation. This has been done previously for CMK-1, and phosphorylated CRH-1 was undetectable (Kimura et al., 2002).

Identifying CRH-1 as a necessary component of long-term memory induction during long-term memory training demonstrates conservation of memory mechanisms across phylogeny regardless of the complexity of the nervous system. An important next step will be to characterize the role of this protein further by confirming which kinase is upstream of CRH-1 activation and how it might alter GLR-1 expression in the posterior ventral nerve cord after training. Knowing the site of plasticity for long-term habituation will greatly facilitate these future studies by allowing us to focus my efforts on candidates that are expressed within AVA, AVD and RIM.

CHAPTER 3: CaMK1 Functions in Adult Learning

3.1 Introduction

In Chapter two I reported that *cmk-1* mutants were unable to form long-term memory for habituation training. To determine whether this memory deficit was specifically due to CMK-1's role in long-term habituation it was important to test if *cmk-1* mutants had normal learning and short-term memory. If *cmk-1* mutants had abnormal learning or short-term habituation it would be more likely that CMK-1 functions in that aspect of learning and not in long-term memory: the long-term memory deficits may only be present because the mutant worms do not learn very well.

Brief and local changes in micromolar concentration levels of free intracellular Ca^{2+} play an important role in modifying many aspects of neural physiology, ranging from neurotransmitter release to long-lasting changes in synaptic strength. Changes in $[Ca^{2+}]$ on this scale are known to activate many Ca^{2+}-sensors, of which an important and ubiquitously expressed protein is Calmodulin (CaM). Once bound with Ca^{2+}, CaM (Ca^{2+}/CAM) is known to regulate many different signaling proteins, including a family of protein kinases that are highly expressed in the nervous system: the CaM-kinases (CaMKs).

Within the CaMK group (consists of ~ 23 kinase families) the CaMK1 family (consisting of CaMKK, CaMK1 and CaMK4; reviewed in Soderling, 1999) has been shown to play important roles in the context of nervous system development and plasticity. CaMKK is purported to function upstream of both CaMK1 and CaMK4 by phosphorylating Thr residues in the activation site of these kinases to increase their Ca/CaM-dependent activities (Haribabu et al., 1995; Kitani et al., 1997). The substrate recognition motifs of numerous protein kinases have significant overlap and correct target specificity is thought to be mediated by localization of the kinase and the target within the cell (Tsui et al., 2005). This is also true of CaMK1 and CaMK4 (Lee et al., 1994). CaMK4's expression is usually restricted to the nucleus (Jensen et al., 1991;

Lemrow et al., 2004; Kotera et al., 2005) where it has been shown to regulate gene transcription during the induction of synaptic plasticity and long-term memory in rodents (Ho et al., 2000a; Kang et al., 2001; Wei et al., 2002). CaMK1's expression, on the other hand, has been observed to be cytoplasmic in most cases (Picciotto et al., 1995), but one exception to this is the beta2 isoform of CaMK1 (Ueda et al., 1999). Many studies have demonstrated an important role for CaMK1 in the developing mammalian nervous system, specifically regulating axonal growth cone motility and axonal outgrowth (Wayman et al., 2004), dendritic arborization (Wayman et al., 2006; Takemoto-Kimura et al., 2007; Wayman et al., 2008b), and formation of dendritic spines and synapses (Saneyoshi et al., 2008).

More recent studies have attempted to ascertain whether CaMK1 plays a role in plasticity of the nervous system: Schmitt et al (2005) investigated the role of CaMKK, CaMK1 and CaMK4 in long-term potentiation (LTP) in rats and demonstrated that CaMKK and CaMK1, but not CaMK4, plays a role in activating Ras-extracellular signal-regulated protein kinase (Ras-ERK) signaling during early-phase LTP in hippocampal neuron cultures. Using the same experimental preparation, Guire et al (2008) went on to further demonstrate that CaMK1 also functions during LTP to recruit calcium permeable AMPA receptors. These studies suggest that similar to CaMK4, CaMK1 also functions in plasticity and perhaps learning and memory, but unlike CaMK4 it most likely functions near the synapse and in shorter forms of plasticity. No one has directly tested if CaMK1 plays a role in plasticity or learning and memory in any organism *in vivo.*

I investigated whether CaMK1 plays a role in adult learning in the nematode *Caenorhabditis elegans*. The *C. elegans* genome encodes a single homologue each of CaMKK, *ckk-1*, and CaMK1, *cmk-1* (Eto et al., 1999). Both genes are expressed in the nervous system and strains with mutations in these genes appear superficially wild-type (Kimura et al., 2002). To test whether or not CaMK1 plays a role in learning I utilized a simple learning assay, habituation to

mechanosensory (tap) stimuli (reviewed in Giles and Rankin, 2008). *C. elegans* respond to tap by performing a reversal (changing from forward to backward locomotion). In wild-type worms, repeated administration of the tap stimulus results in a decrease in both the size of the reversal (response magnitude) elicited by, and the likelihood of responding to (response probability) the tap stimulus (Beck and Rankin, 1993; Sanyal et al., 2004). I hypothesized that if CaMK1 was required for learning then the final habituated level of *cmk-1* mutants would be altered compared to wild-type animals.

I found that *C. elegans* strains carrying mutations in *cmk-1,* but not ckk-1, were deficient at habituating to tap stimuli presented at a 60s ISI. This is the first *in vivo* evidence for the requirement of CaMK in adult plasticity and the first evidence of its requirement for learning in awake behaving animals. A habituation screen of 46 potential downstream targets of CMK-1 suggested by the literature and bioinformatics analysis identified mutations in 4 genes that partially and fully behaviourally phenocopy mutations in *cmk-1*, suggesting they may function downstream of CMK-1 in habituation.

3.2 Methods

3.2.1 Strains and maintenance

Worms were cultured on Nematode Growth Medium (NGM) seeded with *Escherichia coli* (OP50) as described previously (Brenner, 1974). The following strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN): N2 Bristol, VC220 *cmk-1(ok287),* PY1589 *cmk-1(oy21),* VC691 *ckk-1(ok1033),* RB1256 *cdh-4(ok1323),* VC145 *pes-7(gk123),* VC808 *tag-325(ok1330)*, OH4149 *wrk-1(tm1099)*, RB1734 *F47G4.4(ok2219)*, CX51 *dyn-1(ky51)*, RB1625 *par-1(ok2001)*, RB1660 *clec-4(ok2050)*, KU4 *sek-1*(km4), NW990 *unc-129(ev557)*, RB713 *R01H10.7(ok489)*, RB911 *fshr-1(ok778)*, RB1342

ogt-1(ok1474), XA406 *ncs-1(qa406)*, AU1 *sek-1(ag1)*, DA596 *snt-1(ad596)*, FK171 *mek-1(ks54)*, RB653 *ogt-1(ok430)*, RB2108 *inx-11(ok2783)*, VC9 *nca-2(gk5)*, RB676 *num-1(ok433),* RB1323 *C06G1.5(ok1441),* F13G11.1 *dmd-6(gk287),* KG744 *pde-4(ce268),*VC651 *pix-1(ok982),* VC1402 *mef-2(gk633),* CZ3714 *gcy-31(ok296),* NW1255 *seu-1(ev572),* RB758 *hda-4(ok518),* DR26 *daf-16(m26),* RB1439 *F54B3.1(ok1642),* RB1458 *rsr-1(ok1665),* RB1991 *F26F2.1(ok2626),* RB1468 *dkf-2 (ok1704),* RB1826 *T26A5.5(ok2364),* RB2623 *F39B2.7(ok3674),* VC1265 *pyk-1(ok1754),* VC1814 *nhr-275(gk867),* RB1514 *Y71F9B.6(ok1810),* RB2164 *pde-1(ok2924),* VC1299 *R07B7.2(ok1771),* and VC1382 *Y71F9AR.2(ok1893).*

The plasmid containing *Pttx-1*::CMK-1::GFP was a gift from P. Sengupta (Brandeis University, Waltham, MA). The transgenic *C. elegans* strain VH905 hdIs30[*Pglr-1*::DsRed2] was a gift from H. Hutter (Simon Fraser University, Burnaby, BC). The plasmid containing *Pmec-7*::mRFP was a gift from J. Rand (University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma). The transgenic *C. elegans* strains YT1128 *lin-15(n765);* tzEx[*Pckk-1*::GFP; *lin-15*(+)] and YT2016 tzIs2[*Pcmk-1*::GFP; *rol-6(su1006)*] and plasmids containing *cmk-1* cDNA rescues were gifts from Y. Kimura (Mitsubishi Kagaku Institute of Life Sciences, Japan). Full-length genomic *cmk-1* rescues were created by using PCR to amplify the entire *cmk-1* gene from genomic DNA isolated via single-worm DNA lysis. This fragment included ~ 2kb upstream of the predicted ATG start site of the gene. The forward primer used to amplify this fragment was TTAGGCATGGGGTAGGAACTTG, and the reverse primer was GGCACACCCTTTCAGTCAATT. The primers sequences for the PCR fusion construct *Pcmk-1*::GFP were a gift from D. Baillie (Simon Fraser University, Burnaby, BC). The forward and reverse primer sequences used to amplify the *cmk-1* promoter were TATCCAAAATCTTGCCGAAAGTA and agtcgacctgcaggcatgcaagctTAAAAAGGGGGATTGGGC, respectively. The forward and reverse

primer sequences used to amplify GFP were AGCTTGCATGCCTGCAGGTCGACT and AAGGGCCCGTACGGCCGACTAGTAGG, respectively. The forward and reverse primer sequences used to amplify the promoter-GFP fusion construct were AGAATGCCGTATCATAAGCGTAA and GGAAACAGTTATGTTTGGTATATTGGG, respectively.

The following strains were created for this work: VG183 yvEx64[*Pcmk-1*::GFP; *Pmec-7*::mRFP], VG12 hdIs30[*Pglr-1*::DsRed2]; tzIs2[*Pcmk-1*::GFP; rol-6(su1006)], VG19 tzEx[*Pckk-1*::GFP; *lin-15*(+)]; hdIs30[*Pglr-1*::DsRed2], VG92 *cmk -1(oy21)*; yvEx49[*Pcmk-1*::CMK-1; *Pmyo-2*::GFP], and VG100 *cmk -1(oy21)*; yvEx57[*Pcmk-1*::CMK-1; *Pmyo-2*::GFP].

The strain containing the *cmk-1(oy21)* allele was outcrossed 3 times. The strain containing the *ckk-1(ok1033)* allele was outcrossed 6 times. The other strains were listed above were not outcrossed.

3.2.2 Imaging procedures

Adult worms were anesthetized in 100 mM NaN_3 dissolved in M9 buffer containing sephadex beads (to prevent the worms from being crushed by the coverslip; G-150-50, Sigma-Aldrich, St. Louis, MO) on glass microscope slides, and then covered with a 1.5 thickness coverslip. An Olympus Fluoview 1000 Confocal microscope was used for imaging. GFP was excited using a 488 nm wavelength laser setting with light emitted collected through a 491-515 nm bandpass filter. dsRed and mRFP were excited using a 543 nm wavelength laser setting with light emitted collected through a 600-630 nm bandpass filter. Optical sections of 0.5 μm thickness were collected using a 60x oil immersion lens (Olympus). All images were of a 1024 X 1024 aspect ratio and were composed of 10-30 serial sections. Final figures were generated using Image J version 1.41o (National Institutes of Health, Bethesda, MD) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

3.2.3 Behavioural testing of mutant strains

Worms were synchronized for behavioural testing by picking 5 gravid adults onto a Petri plate containing nematode growth media (NGM) seeded with 100 μl of a liquid culture of OP50 *E. coli* 12-24 hours earlier and letting them lay eggs for 3-4 hrs before they were removed. These eggs were allowed to develop for 72-120 hours (dependent upon the experiment) in a closed Tupperware box in a 20°C incubator. Plates of worms were placed into the tapping apparatus (Swierczek et al., 2011) and covered with an optically transparent lid constructed from a Petri plate lid, non-fogging cover-glass and wax. After a 100 s acclimatization period, 30 taps were administered at either a 60s or a 10s ISI.

3.2.4 Behavioural testing of CMK-1 rescue strains

Worms were synchronized for behavioural testing by selecting 5 gravid adults carrying the selection marker for the rescue DNA (GFP) onto an OP50 *E. coli* seeded plate and letting them lay eggs for 3-4 hrs before they were removed. These eggs were allowed to develop for 96 hours in a closed Tupperware box in a 20°C incubator. Twelve hours prior to testing, 40-60 worms carrying the selection marker were transferred using a platinum pick to a fresh NGM plate. Plates were seeded with 50 μl of a liquid culture of OP50 *E. coli* 16-20 hours beforehand. Plates of worms were placed into the tapping apparatus (Swierczek et al., 2011) and covered with an optically transparent lid constructed from a Petri plate lid, non-fogging cover-glass and wax. After a 100 s acclimatization period, 30 taps were administered at either a 60s or a 10s ISI.

3.2.5 Image acquisition of behaviour

Stimulus delivery and image acquisition to record the behaviour of the worms was done using the MWT (version 1.2.0.2) (Swierczek et al., 2011). A Dalsa Falcon 4M30 camera (8 bits;

2352 x 1728 pixels, 31 Hz) and a Rodenstock 60 mm f-number 4.0 Rodagon lens was used to visualize a 5-cm Petri plate secured in the tapping apparatus. Images from the camera were captured using a National Instruments PCIe-1427 CameraLink capture card run along with the MWT tracking software on PC computers with Intel Core i3 2.93 GHz processors and 4 GB of RAM. The image of the plate was focused into the camera with a resolution of 0.027 mm/pixel. An elliptical region of interest was created ~5 mm from the inner edge of the Petri plate. Minimum and maximum object size thresholds were 80 and 400 pixels, respectively. The position, skeleton and outline of worms were acquired at a rate of 25 frames per second.

3.2.6 Behavioural scoring and statistical analysis

Offline data analysis was performed on a computer with a Intel Core i7-930 2.80 GHz processor with 6 GB of RAM using Choreography analysis software (version 1.3.0_r1035 software package) (Swierczek et al., 2011) to detect and measure the distance traveled during tap-evoked reversals. Choreography options "--shadowless", "--minimum-time 20", and "--minimum-move-body 2" were applied as filters. "--segment", "--plugin Reoutline", and "--Respine" were used to enhance detection of worm direction. "—plugin MeasureReversal::tap::dt=1::collect=0.5" was used to detect reversals within 1s of taps.

Two measures were analyzed: reversal probability (the number of worms that responded to tap with a reversal from all plates within the same experimental conditions summed and divided by the sum of the number of worms reversing and the number that did not respond) and reversal magnitude (reversal distance measured for worms that responded within 1 second to tap). For both measures worms that were already reversing at the onset of the stimulus were omitted.

Reversal distances in response to either the initial or final tap of worms of each genotype were compared by statistical analysis of covariance (ANCOVA) and *post hoc* Tukey honestly

significant difference (HSD) tests. Genotype was modeled as a fixed effect. Petri plate (on which the worms were tested; minimum of 3 Petri plates of ~ 50 worms per experimental condition) was modeled as a random effect nested within the fixed effect. The relationship between genotype and reversal probability and in response to either the initial or final tap was analyzed using a generalized linear mixed-model regression (GLMM). Similar to the statistical analysis of reversal distance, plate was again modeled as a random effect. Additive modeling was performed and the genotype of wild-type (and *cmk-1(oy21)* for rescue experiments) was used as a baseline to compare against the remaining groups. For all statistical tests an alpha value of 0.05 was used to determine significance. Statistical analyses for initial response probability and reversal distance was only performed for 60s ISI experiments because the initial response probability and reversal distance data for 10s ISI experiments was considered to be equivalent and redundant. Matlab (version R2010b, Mathworks, Natick, MA, USA) was used for plotting graphs. ANCOVAs, Tukey's HSD post-hoc tests and mixed-model logistic regressions were performed using the statistical packages lme and glmmPQL in R (for Mac OS X GUI 1.40-devel Leopard build 32-bi).

3.3 Results

3.3.1 Short-term habituation of *cmk-1* and *ckk-1* mutants

To determine whether two components of the CaMK cascade (CMK-1 and CKK-1) play a role in the tap withdrawal response itself and/or habituation I administered 30 taps at both a 60 and a 10s ISI to wild-type, *cmk-1(oy21), cmk-1(ok287) and ckk-1(ok1033)* mutants 96 hours post-egg lay. The *cmk-1(oy21)* allele is a nonsense Q to amber stop (127) mutation in the third exon and is hypothesized to be a null (Satterlee et al., 2004). The *cmk-1(ok287)* allele is a 1957 bp deletion that removes a large portion of the kinase catalytic domain (exons 3, 4, 5 and part of exon 6) and thus is likely a null. The *ckk-1(ok1033)* allele is a 1067 bp deletion that removes

about 80% of the kinase catalytic domain (exons 4, 5, 6 of the CKK-1A splice variant and exons 2, 3, 4 of the CKK-1B splice variant) and thus is also likely a null.

I chose to investigate habituation at both a 60 and 10s ISI because behavioural studies have shown that rate and asymptotic level of habituation are dependent on frequency of stimulation and are likely mediated be different molecular mechanisms (Rankin and Broster, 1992). I also analyzed the behavioural metrics of response probability and reversal distance in response to tap separately because a recent study by our lab has shown that habituation of response probability and reversal distance habituation are mediated by different mechanisms at least at a 10s ISI (Giles et al., 2011).

Reversal Distance: *cmk-1(oy21)* mutants responded to the initial tap with significantly larger reversals than did wild-type worms (figure 3.1A, ANCOVA: $F(3,23)=7.90$, $p=0.0008$, Tukey's HSD: $p<0.001$). The reversal distance in response to the initial tap of the other mutants was statistically indistinguishable from that of the wild-type worms (Tukey's HSD: pNS).

When worms were habituated to taps delivered at a 60s ISI both *cmk-1(oy21)* and *cmk-1(ok287)* mutants responded to the final tap with significantly larger reversals than did wild-type worms (figure 3.1A, ANCOVA: $F(3,23)=14.20$, $p<0.0001$, Tukey's HSD: $p<0.001$ for both mutants). Interestingly, the reversal distance of the final response to tap of *ckk-1(ok1033)* was statistically indistinguishable from that of the wild-type worms (Tukey's HSD: pNS). In contrast, when worms were habituated to taps delivered at a 10s ISI only *cmk-1(ok287)* mutants responded to the final tap with significantly larger reversals than did wild-type worms (figure 3.1B, ANCOVA: $F(3,16)=4.29$, $p<0.02$, Tukey's HSD: $p=0.02$ for both mutants). In this experiment

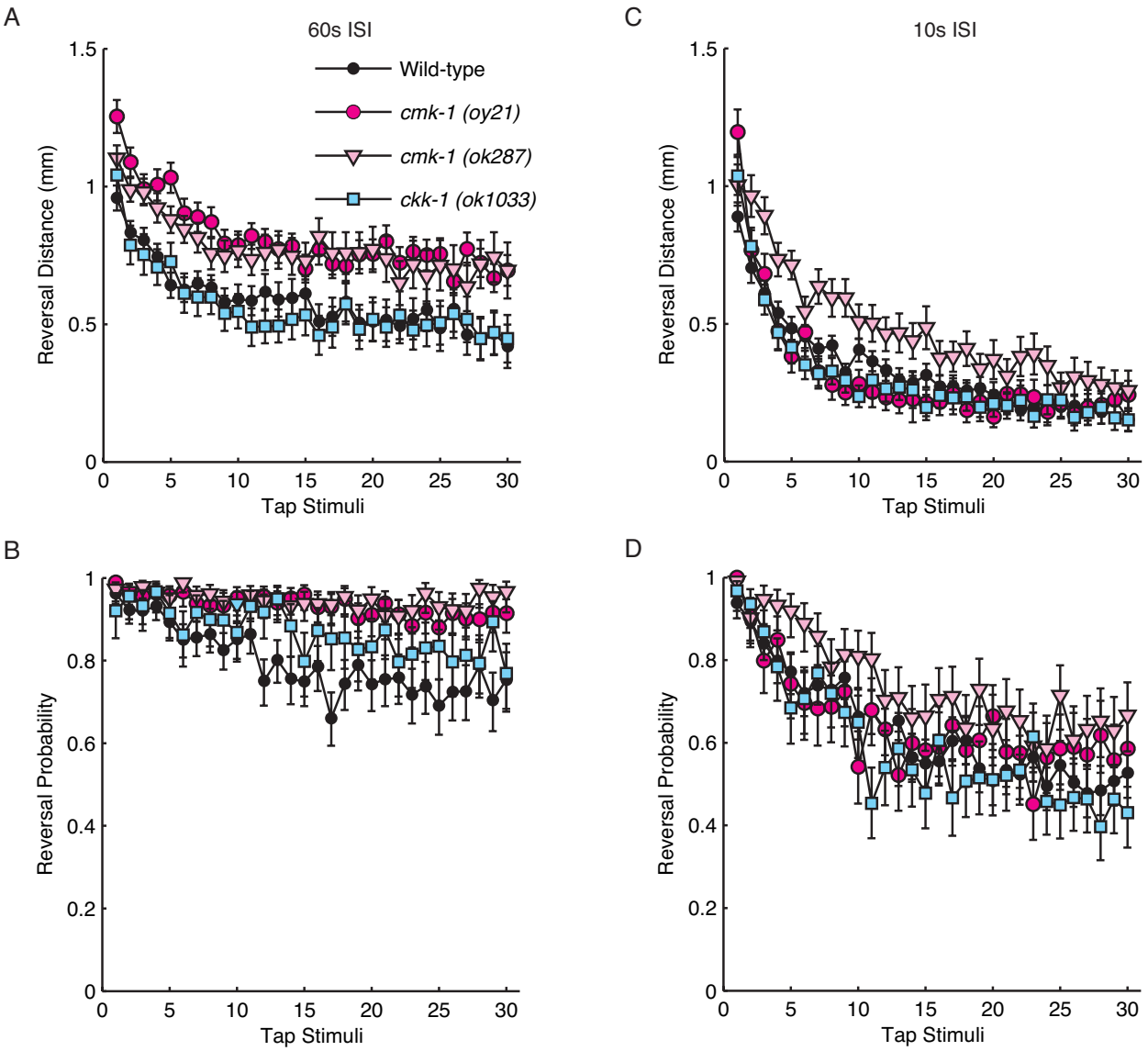

Figure 3.1 Tap habituation curves of 96 hour-old wild-type, cmk-1 and ckk-1(ok1033) mutant *C. elegans* in response to a series of 30 taps at a 60s and 10s ISI.

Tap elicits larger reversals from naive *cmk-1(oy21*) mutants compared to naïve wild-type worms and both *cmk-1* mutants respond with significantly larger reversals than wild-type after habituation training at a 60s ISI. (A). All worm genotypes tested were equally likely to respond to the initial tap stimuli, but both alleles of *cmk-1* were less likely to respond to the last tap of a habituation series of 30 taps at a 60s ISI (B). After habituation at a 10s *ISI cmk-1 (ok287*) mutants were responding with significantly larger reversals than wild-type worms (C). No difference between genotypes was observed in habituation of response probability when worms were stimulated at a 10s ISI (D). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

cmk-1(oy21) and *ckk-1(ok1033)* mutants were statistically indistinguishable from the wild-type worms (Tukey's HSD: pNS).

Response Probability: All mutants were as likely as wild-type worms to respond to the initial tap (figure 3.1C, pNS for all genotypes).

When worms were habituated to taps delivered at a 60s ISI (figure 2.1C) both *cmk-1(oy21)* and *cmk-1(ok287)* mutants were significantly more likely (p=0.03 and p=0.004, respectively) to respond to the final tap of a habituation series than were wild-type worms, but *ckk-1(ok1033)* mutants were statistically indistinguishable from the wild-type worms (pNS). In contrast, when worms were habituated to taps delivered at a 10s ISI all mutants were as likely as wild-type worms to respond to the final tap (figure 3.1D, pNS for all genotypes).

Thus, CMK-1 but not CKK-1, appears to play an important role in habituation at a 60s ISI as both mutant alleles of *cmk-1* showed shallower habituation compared to wild-type worms in both reversal distance and response probability measures. Neither gene appears to be critical for habituation at a 10s ISI.

3.3.2 Expression of CMK-1 and CKK-1

I found that only mutations in *cmk-1*, but not *ckk-1*, caused obvious alterations in the tap withdrawal response and habituation and so I investigated whether or not CMK-1 and CKK-1 were expressed in the sensory neurons and interneurons of the tap withdrawal circuit. To do this I used transgenes expressing a reporter construct (GFP, dsRed or mRFP) under the control of the putative promoter to assess where the gene that follows that promoter in the genome is expressed. Both MEC-7 expressing cells (marker of mechanosensory neurons) and GLR-1 expressing cells (marker of interneurons, including the tap withdrawal interneurons: AVA, AVB, AVD and PVC) were found to express CMK-1 (figure 3.2A, B; PVC data not shown). In contrast, GLR-1 expressing cells (figure 3.2C) and the mechanosensory neurons (figures 2D &

E; see also Kimura et al 2002) did not express CKK-1. My data are consistent with previous findings that the expression profile of CMK-1 was much broader than the expression profile of CKK-1 (Kimura et al., 2002). These expression data also match the behavioural data and support a role for CMK-1, but not CKK-1, in the tap withdrawal response and habituation.

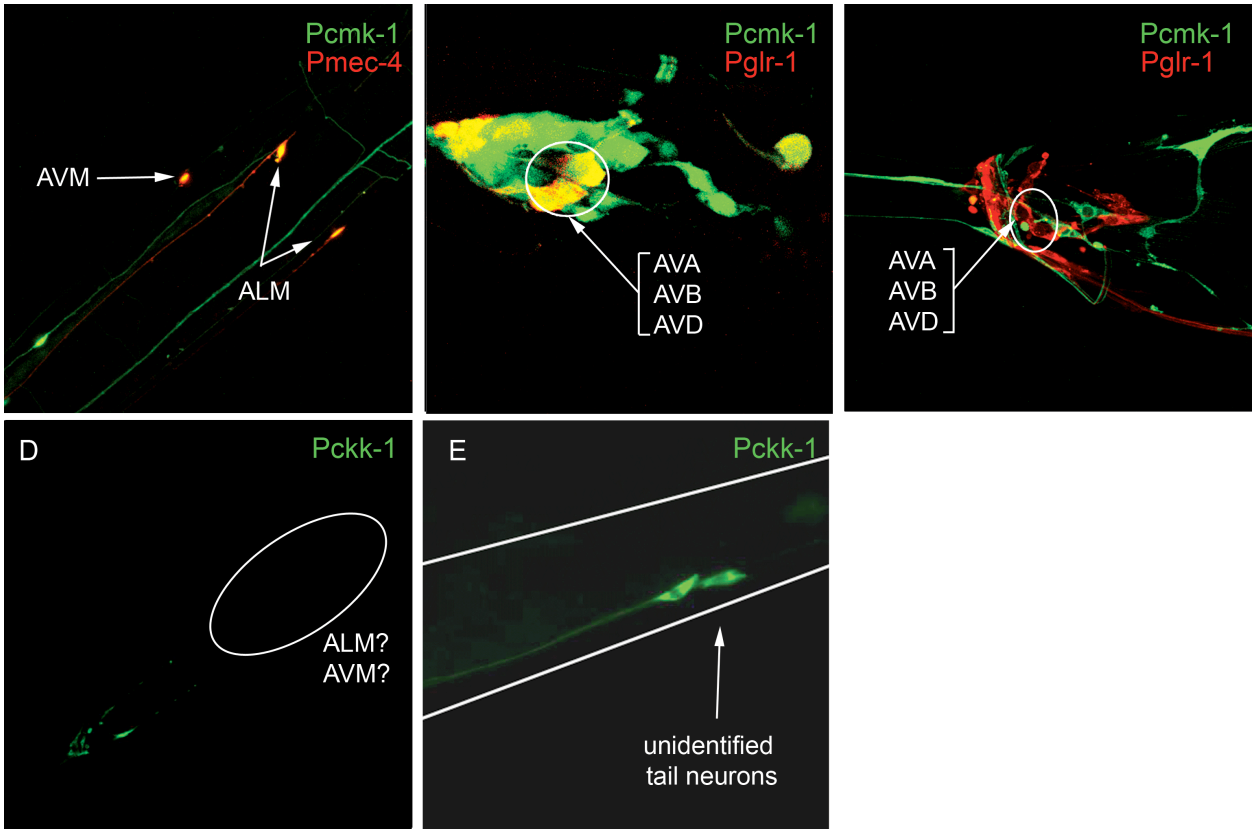

Figure 3.2 CMK-1 but not CKK-1 is expressed in the sensory and interneurons of the tap withdrawal circuit.

A) Mechanosensory neurons (visualized with *Pmec-7*::mRFP) express CMK-1 (visualized with *Pcmk-1*::GFP). B) tap withdrawal interneurons, AVA, AVB, AVD (visualized with *Pglr-1*::dsRed), express CMK-1 (visualized with *Pcmk-1*::GFP). C) GLR-1 expressing neurons (including the tap withdrawal interneurons, AVA, AVB, AVD; visualized with Pglr-1::dsRed) do not express CKK-1 (visualized with *Pckk-1*::GFP). D) No fluorescence (visualized with *Pckk-1*::GFP) was observed where ALM and AVM cell bodies are located (between the nerve ring and the vulva. E) A pair of neuronal cell bodies was observed to fluoresce in the tail, but because their neurites projected directly into the ventral nerve cord they cannot be PLM (PLM neurites project anteriorly along the lateral sides of the body before sending a projection down to the ventral nerve cord).

3.3.3 Habituation of *cmk-1* mutants in younger and older worms

To investigate whether the deficit of *cmk-1* mutant worms was dependent on the age the worms I administered 30 taps at both a 60 and 10s ISI *cmk-1(oy21)* and wild-type worms at both younger and older time points (72 and 120 hours post-egg lay, respectively). I chose to use the *cmk-1(oy21)* allele because the *oy21* mutation results in deletion of a greater portion of the protein than does the *ok1033* allele.

Reversal Distance: The reversal distance in response to the initial tap of 72 hour-old *cmk-1(oy21)* mutant worms was were statistically indistinguishable from 72 hour-old wild-type worms (figure 3.3A, ANCOVA: $F(1,8)=4.39$, pNS).

When 72 hour-old worms were habituated to taps delivered at a 60s ISI, *cmk-1(oy21)* mutants responded to the final tap with significantly larger reversals than did wild-type worms (figure 3.3A, ANCOVA: $F(1,8)=16.54$, $p<0.004$, Tukey's HSD: $p<0.0001$). In contrast, when 72 hour-old worms were habituated to taps delivered at a 10s ISI the reversal distance of the final response to tap of *cmk-1(oy21)* mutant and wild-type worms were statistically indistinguishable (figure 3.3B, ANCOVA: $F(1,10)=3.55$, pNS).

Response Probability: 72 hour-old *cmk-1(oy21)* mutants were as likely as 72 hour-old wild-type worms to respond to the initial tap (figure 3.3A, pNS).

When 72 hour-old worms were habituated to taps delivered at a 60s ISI (figure 3.3C), *cmk-1(oy21)* mutants were significantly more likely than wild-type worms to respond to the final tap of a habituation series ($p=0.04$). In contrast, when worms were habituated to taps delivered at a 10s ISI, the probability of responding to the final response to tap of 72 hour-old worms was not statistically indistinguishable between *cmk-1(oy21)* mutant and wild-type worms (figure 3.3D, pNS).

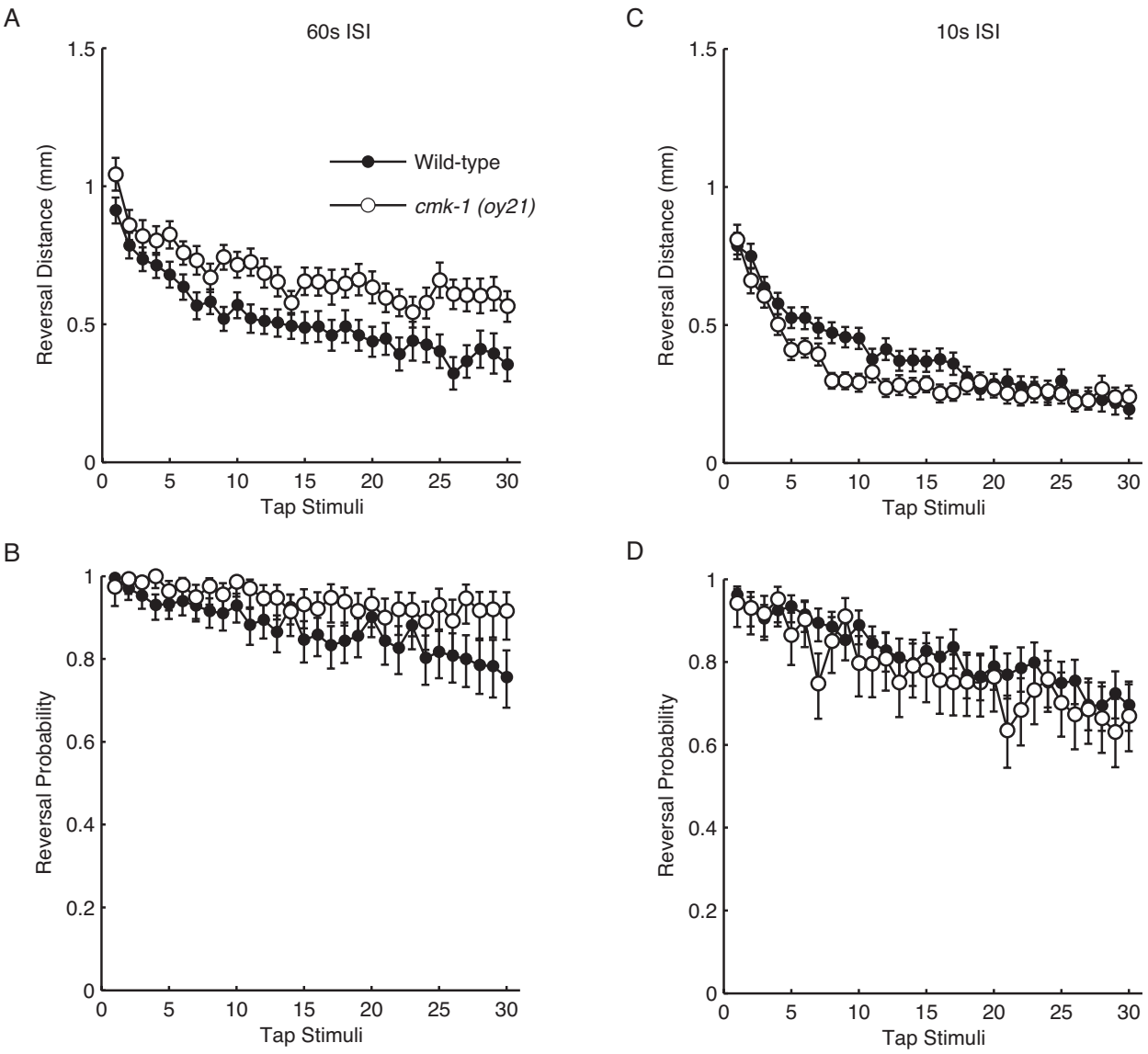

Figure 3.3 Tap habituation curves of 72-hour old wild-type and *cmk-1(oy21)* mutant *C. elegans* in response to a series of 30 taps at a 60s and 10s ISI.

No difference was observed in reversal difference from naive cmk-1 (oy21) mutants compared to naïve wild-type worms and cmk-1 mutants respond with significantly larger reversals than wild-type after habituation training at a 60s ISI (A). *cmk-1* mutants were less likely to respond to the last tap of a habituation series of 30 taps at a 60s ISI (B). No difference between genotypes was observed in habituation of reversal distance (C) or response probability when worms were stimulated at a 10s ISI (D). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

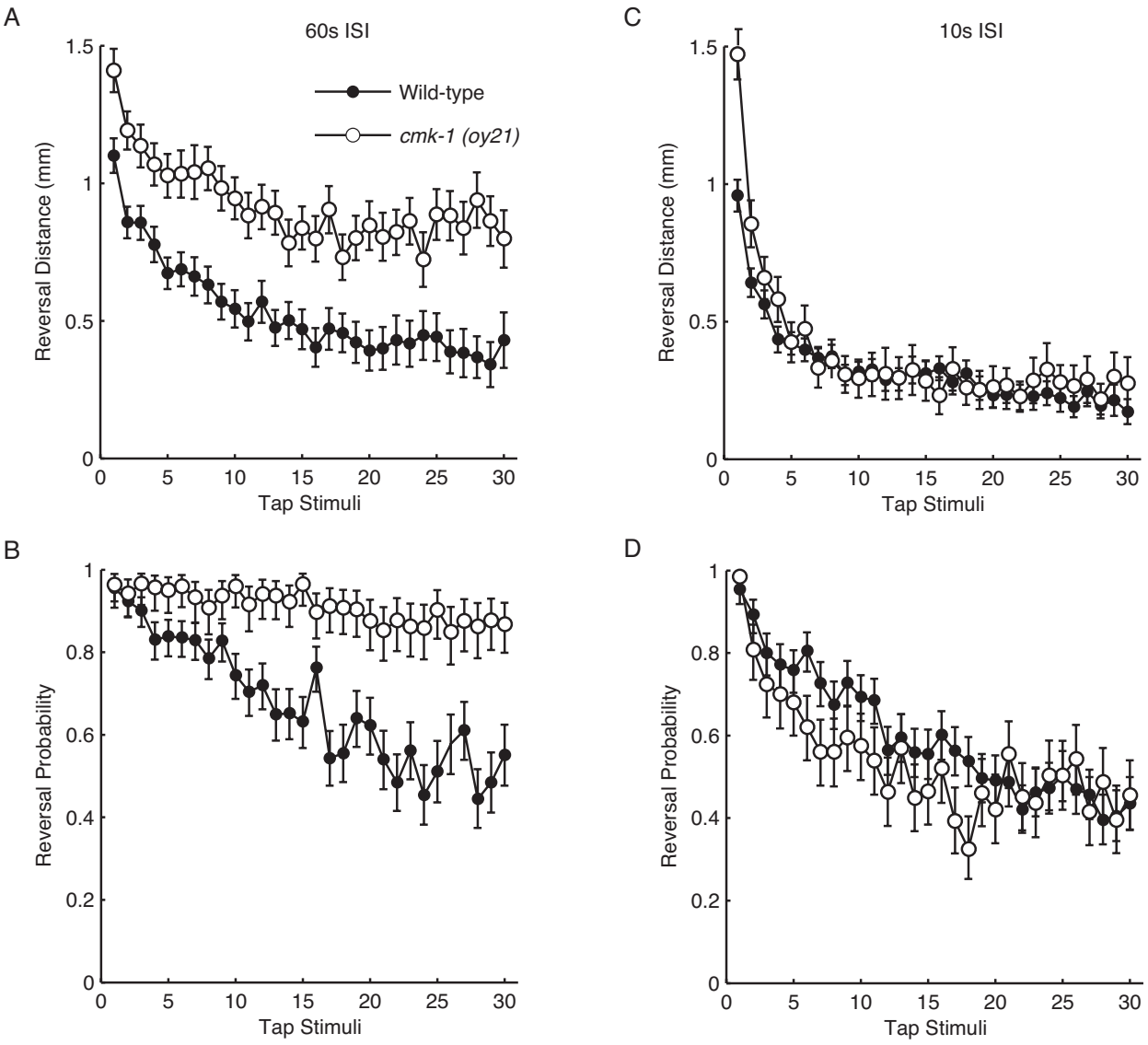

Figure 3.4 Tap habituation curves of 120-hour old wild-type and cmk-1 (oy21) mutant *C. elegans* in response to a series of 30 taps at a 60s and 10s ISI.

Tap elicits larger reversals from naive *cmk-1(oy21)* mutants compared to naïve wild-type worms and *cmk-1* mutants respond with significantly larger reversals than wild-type after habituation training at a 60s ISI (A). *cmk-1* mutants were less likely to respond to the last tap of a habituation series of 30 taps at a 60s ISI (B). No difference between genotypes was observed in habituation of reversal distance (C) or response probability when worms were stimulated at a 10s ISI (D). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

Reversal Distance: 120 hour-old *cmk-1(oy21)* mutants responded with a significantly larger reversal distance in response to the initial tap compared to 120 hour-old wild-type worms (figure 3.4A, ANCOVA: $F(1,10)=42.81$, $p=0.0001$, Tukey's HSD: $p<0.0001$).
When 120 hour-old worms were habituated to taps delivered at a 60s ISI, *cmk-1(oy21)* mutants responded to the final tap with significantly larger reversals than did wild-type worms (figure 3.4A, ANCOVA: $F(1,10)=36.12$, $p=0.0001$, Tukey's HSD: $p<0.0001$). In contrast, when 120 hour-old worms were habituated to taps delivered at a 10s ISI, there were no statistically significant differences between *cmk-1(oy21)* and wild-type worms in habituated level of reversal distance (figure 3.4B, ANCOVA: $F(1,10)=2.94$, pNS).

Response Probability: 120 hour-old *cmk-1(oy21)* mutants were as likely as 120 hour-old wild-type worms to respond to the initial tap (figure 3.4C, pNS).

When 120 hour-old worms were habituated to taps delivered at a 60s ISI (figure 3.4C), *cmk-1(oy21)* mutant were significantly more likely than wild-type worms to respond to the final tap of a habituation series ($p=0.002$). In contrast, when worms were habituated to taps delivered at a 10s ISI, the probability of responding to the final response to tap of 120 hour-old worms was not statistically indistinguishable between *cmk-1(oy21)* mutant and wild-type worms (figure 3.4D, pNS).

Thus in 72, 96 and 120 hr-old worms *cmk-1* mutants habituate normally at a 10s ISI, but in all three ages (72, 96 and 120 hr-old worms) they show shallower habituation than wild-type worms at a 60s ISI in both behavioural measures. These experiments suggest that only CMK-1 and not CKK-1 appears to be important for habituation of response magnitude and probability at a 60s ISI. CMK-1, but not CKK-1, may also play a role at a 10s ISI, but this may be a more minor role than at a 60s ISI because this phenotype was only observed in 96 hr-old worms in one of two mutant strains and then, only in the measure of reversal distance. Because of this I chose

to follow-up the role of CMK-1 in habituation at a 60s ISI only.

3.3.4 CMK-1 cDNA rescues habituation phenotypes in *cmk-1* mutants

To ensure that the decrease in the ability of *cmk-1* mutants to habituate normally at a 60s ISI was due to the identified mutation in *cmk-1* and not due to other background mutations in the strain I expressed the CMK-1 cDNA under control of its endogenous promoter in *cmk-1(oy21)* mutants (*cmk-1(oy21);* CMK-1) and tested their habituation at a 60s ISI (figure 3.5).

Reversal Distance: A partial rescue of the *cmk-1* initial tap reversal distance phenotype in the mutants expressing a wild-type copy of the CMK-1 cDNA was observed: the initial responses of *cmk -1(oy21)*; yvEx49 and *cmk -1(oy21)*; yvEx57 were statistically indistinguishable from wild-type and *cmk-1(oy21)* mutants (figure 3.5A, ANCOVA: $F(3,17)=4.14$, $p=0.02$, Tukey's HSD: pNS for both comparisons).

When worms were habituated to tap at a 60s ISI I observed a full rescue of the *cmk-1* reversal distance habituation phenotype in the mutants expressing a wild-type copy of the CMK-1 cDNA: *cmk -1(oy21)*; yvEx49, *cmk -1(oy21)*; yvEx57 and wild-type worms responded to the final tap with significantly smaller reversals than did *cmk-1(oy21)* mutants (figure 3.5A, ANCOVA: $F(3,17)=3.92$, $p=0.03$, Tukey's HSD: $p=0.04$, $p=0.03$ and $p=0.01$, respectively).

Response Probability: A full rescue of the response probability habituation phenotype was seen for *cmk -1(oy21)*; yvEx49: this rescue line's probability of responding the final tap was statistically indistinguishable from wild-type (pNS) and was significantly less likely to respond to the final tap of a habituation series (60s ISI) than were *cmk-1(oy21)* mutants ($p=0.04$). No rescue of the response probability habituation phenotype was seen for *cmk -1(oy21)*; yvEx57 as this rescue line was significantly more likely to respond to the final taps than wild-type worms ($p=0.005$) and statistically indistinguishable from *cmk-1(oy21)* mutants (pNS).

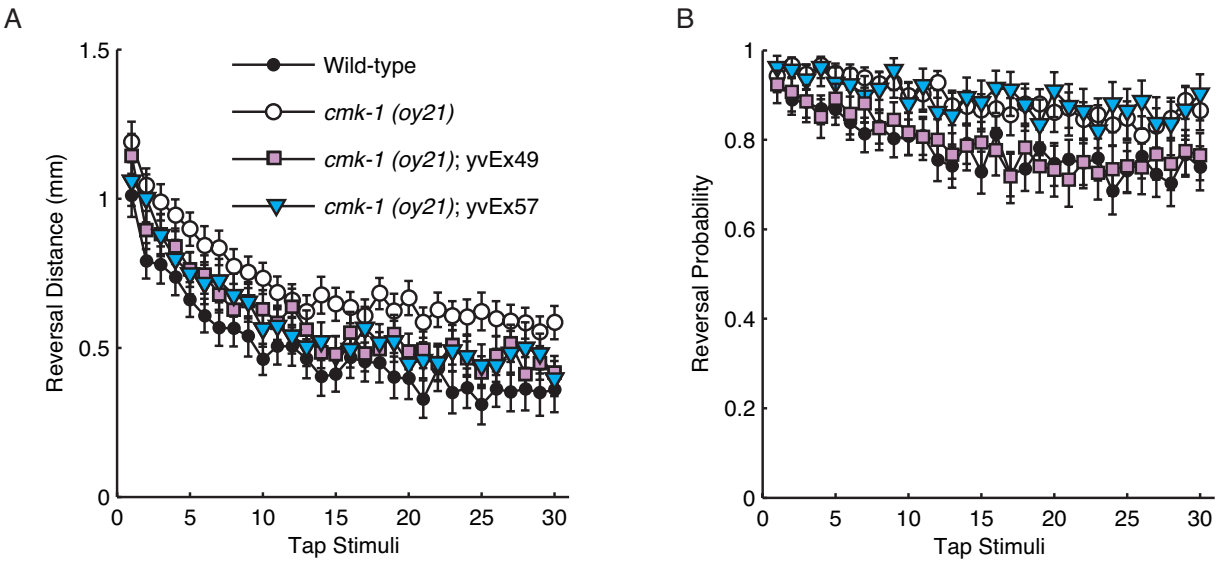

Figure 3.5 Tap habituation curves of 96-hour old wild-type and *cmk-1 (oy21)* mutant and 2 CMK-1 rescue strains *C. elegans* in response to a series of 30 taps at a 60s ISI.

cmk-1 mutants respond with significantly larger reversals than wild-type and the two CMK-1 rescue strains after habituation training at a 60s ISI (A). *cmk-1* mutants and were less likely to respond to the last tap of a habituation series of 30 taps at a 60s ISI than wild-type and the *cmk-1(oy21)*; yvEx49 rescue strain (B). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

Thus I found one CMK-1 rescue line (*cmk -1(oy21)*; yvEx49) that rescued both the reversal distance and response probability habituation phenotypes and one line (*cmk -1(oy21)*; yvEx57) that rescued only the reversal distance habituation phenotype. These data strongly suggest that the habituation deficits seen at a 60s ISI in *cmk-1* mutants are due to the identified mutations in *cmk-1* and not to other background mutations within the strains.

3.3.5 A screen for phosphorylation targets of CMK-1

To find the downstream targets of CMK-1 in habituation at a 60s ISI I reviewed the literature for previously identified phosphorylation targets and used bioinformatics to predict novel phosphorylation targets and then screened worm strains with mutations in these genes for habituation defects at a 60s ISI. Similar to other Ser/Thr protein kinases, CMK-1 selects the specific Ser/Thr amino acid residues to phosphorylate on its target substrates based on the

sequence of residues that flank the phosphoacceptor site (comprises the kinase consensus sequence). I was given consensus sequences for the human orthologs of CMK-1 (CaMK1a, 1d, 1g and CaMK4) predicted by bioformatics as a gift from S. Pelech (University of British Columbia). I used protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to search for occurrence of these consensus amino acid sequences in the *C. elegans* genome. X was used to denote amino acids that were not predictive/important for the consensus sequences. Because some amino acids in the consensus sequence are known to be more predictive/important for target recognition I omitted protein BLAST results that did not include these amino acids. A review of the literature resulted in the identification of 30 known phosphorylation targets (Table 3.1), and protein BLAST of the human CaMK consensus sequences against the *C. elegans* genome resulted in the identification of 246 potential targets of CMK-1 (Table 3.2).

From this list of targets I selected genes to test for habituation at a 60s ISI that 1) had worm strains with mutations in these genes available at the *Caenorhabditis* Genetics Centre that lived to be viable adults, 2) were expressed in the nervous system (or at least had not been demonstrated to not be expressed there), and 3) showed no obvious motor abnormalities or developmental delays. In total I tested 46 mutant *C. elegans* strains. All mutants were 96 hour post-egg lay when tested. Results from the screen are graphed in figure 3.6, statistical analysis is reported in table 3.3 (p values reported represent comparison against the wild-type strain ran on the same day, + represents a phenotypic response greater than wild-type worms, whereas – represents a smaller phenotypic response than wild-type worms) and a Venn diagram grouping genes whose mutant alleles showed similar behavioural phenotypes is presented in figure 3.7. Four mutants phenocopied *cmk-1* mutants to various extents: *cdh-1(ok1323), snt-1(ad596), ogt-1(ok430),* and a homologue to the human GTP binding protein 3 (GTPBP3), *F39B2.7(ok3674).*

cdh-1(ok1323) mutants phenocopied *cmk-1* mutants in the measures reversal distance in response to the initial tap (figure 3.6A, larger responses than wild-type worms, $p<0.01$) and in

the probability of responding to the final tap in a habituation series (figure 3.6B, more likely to respond than wild-type worms, $p<0.05$). In the measures of reversal distance of habituated level and probability of responding to the initial tap they were statistically indistinguishable from wild-type worms (pNS for both).

snt-1(ad596) mutants phenocopied *cmk-1* mutants in the measures reversal distance in response to the initial tap (figure 3.6I, larger responses than wild-type worms, $p<0.01$) and in the size of the reversal distance in response to the final tap in a habituation series (figure 3.6J, larger responses than wild-type worms, $p<0.05$). In both measures of reversal probability (likelihood of responding to the initial and final tap) they were statistically indistinguishable from wild-type worms (pNS for both).

ogt-1(ok430) mutants only phenocopied *cmk-1* mutants in one measure; they gave statistically larger reversals in response to the initial tap (figure 3.6G, $p<0.05$). And similar to *cmk-1* mutants, in the measures of likelihood of responding to the initial tap they were statistically indistinguishable from wild-type worms (pNS). Unlike *cmk-1* mutants, *ogt-1(ok430)* mutants were statistically indistinguishable from wild-type worms in their response to the final tap in the measure of reversal distance (pNS) and in the measure of response probability (figure 3.6H, pNS). But, I also tested a second allele of *ogt-1* and found that *ogt-1(ok1474)* mutants phenocopied *cmk-1* mutants in all measures; they gave statistically larger reversals in response to the initial and final taps (figure 3.6I, $p<0.0001$ for both) and were more likely to respond the final tap in a habituation series (figure 3.6J, $p<0.01$). And similar to *cmk-1* mutants, in the measures of probability of responding to the initial tap they were statistically indistinguishable from wild-type worms (pNS).

F39B2.7(ok3674) mutants phenocopied *cmk-1* mutants in the size of the reversal distance in response to the final tap in a habituation series (figure 3.6U, larger responses than wild-type worms, $p<0.05$) and in the probability of responding to the final tap in a habituation series

(figure 3.6V, more likely to respond than wild-type worms, $p<0.01$). Unlike *cmk-1* mutant worms *F39B2.7(ok3674)* mutants were more likely to respond the initial tap than wild-type worms ($p<0.0001$) and were statistically indistinguishable from wild-type worms in the measure of reversal distance to the initial tap (pNS).

Table 3.1 Known downstream targets of CaMK1 and CaMK4.

Gene Name	Evidence	C. elegans homologue	C. elegans Neuronal Expression?	Phosphorylated by
AMPK (AMP-activated protein kinase)	Kim et al., 2009	aak-1, aak-2	Yes	CaMK4
p300 (transcriptional co-activator)	Corcoran et al., 2003; Yuan et al., 2002	cbp-1	Yes	CaMK1, CaMK4
cAMP response element binding protein (CREB)	Sheng et al., 1991; Chatila et al. 1996	crh-1	Yes	CaMK1
ATF1 (activating transcription factor)	Sun et al., 1996	crh-1	Yes	CaMK1
VDR (vitamin D receptor)	Ellison et al., 2005	daf-12	Yes	CaMK4
Drp1 (dynamin-related protein 1)	Han et al., 2008	drp-1, dyn-1	Yes	CaMK1
LIM kinase 1 (LIMK1)	Takemura et al., 2009	gck-1	?	CaMK4
GADPH (Glyceraldehyde 3-phosphate dehydrogenase)	Ishida et al., 2004	gdp-1, gdp-2, gdp-3, gdp-4	Yes	CaMK1
HDAC-5 (histone deacetylase-5)	McKinsey et al., 2000	hda-4	Yes	CaMK1
HMGB-1 (high-mobility group box 1 chromatin-binding factor)	Zhang et al., 2008	hmg-5	Yes	CaMK4
HSP-25 (25 kDa heat shock protein)	Corcoran et al., 2003	hsp-25	No	CaMK1
eIF4GII (eukaryote translation initiation factor 4GII)	Qin et al., 2003	ifg-1	Yes	CaMK1
MEF2 (myocyte enhancer factor-2)	Blaeser et al., 2000; Passier et al., 2000	mef-2	Yes	CaMK4
MRLC (myosin II regulatory light chain)	Suizu et al., 2002	mlc-1, mlc-2, mlc-4	No	CaMK1
Erk and MAP Kinases	Schmitt et al., 2004, Enslen et al., 1996; Wayman et al., 2006	mpk-1, mek-1, mek-2, sek-1	Yes	CaMK1, CaMK4
CFTR (cystic fibrosis transmembrane conductance regulator)	Picciotto et al., 1992	mrp-5	Yes	CaMK1
FRQ (Frequency)	Yang et al., 2001	ncs-1	Yes	CaMK1
Numb/Numbl	Tokumitsu et al., 2005; Tokumitsu et al., 2006	num-1	Yes	CaMK1, CaMK4
OGT (O-linked N-acetylglucosamine (O-GlcNAc) transferase)	Song et al., 2008	ogt-1	Yes	CaMK4

Gene Name	Evidence	C. elegans homologue	C. elegans Neuronal Expression?	Phosphorylated by
MARKS2/Par-1b	Uboha et al., 2007	par-1	Yes	CaMK1
βPix	Saneyoshi et al., 2008	pix-1	No	CaMK1
RPS19 (ribosomal protein S19)	Maeda et al., 2009	rps-19	?	CaMK1
p65/synaptotagmin	Jang et al., 2001	snt-1	Yes	CaMK4
PFK-2 (Phosphofructokinase 2)	Qin et al., 2003	Y110A7A.6	?	CaMK1
cardiac phospholamban peptide	Uemura et al., 1998	none exist	Not Applicable	CaMK1
cabin-1 (calcineurin-binding protein 1)	Pan et al., 2005	none exist	Not Applicable	CaMK4
NOS (nitric-oxide synthase)	Song et al., 2004	none exist	Not Applicable	CaMK1
Op18/stathmin	Holmfeldt et al., 2007	none exist	Not Applicable	CaMK4
protamine-2	Wu et al., 2000	none exist	Not Applicable	CaMK4

Table 3.2 Putative downstream targets of CaMK1 and CaMK4 predicted by bioinformatics

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
acdh-8	K05F1.3	Medium-chain acyl-CoA dehydrogenase	?	CaMK1g
aqp-5	C35A5.1	aqp-5 encodes a putative aquaporin with no known substrate	Yes	CaMK1b
brc-1	C36A4.8a	brc-1 encodes an ortholog of human BRCA required for double-strand break repair via inter-sister recombination during meiosis	?	CaMK1b
cdh-4	F25F2.2	A homolog of a member of the cadherin superfamily that is involved in cell-cell adhesion	Yes	CaMK1a, 1d
clec-4	Y38E10A.5	C-type lectin	?	CaMK1a, 1d
csr-1	F20D12.1	Translation initiation factor 2C (eIF-2C) and related proteins	?	CaMK1a, 1d
cut-6	M142.2		?	CaMK1g
cutl-7	F53B6.6		?	CaMK1a, 1d
daf-16	R13H8.1	a transcription factor of the HNF-3/forkhead family that acts in an insulin-mediated pathway	Yes	CaMK1g
daf-2	Y55D5A.5	a predicted receptor tyrosine kinase that is the C. elegans insulin/IGF receptor ortholog	Yes	CaMK4
dep-1	F44G4.8	a class III receptor protein tyrosine phosphatase (R-PTP) orthologous to human R-PTPs such as PTPRJ/Dep-1	?	CaMK1g
dkf-2	T25E12.4	encodes one of two C. elegans novel protein kinase Ds	Yes	CaMK1a, 1b, 1d
dmd-6	F13G11.1	Transcription factor Doublesex	?	CaMK1a, 1d
dmd-6	F13G11.1c	Transcription factor Doublesex [KOG3815]	?	CaMK1d
dmd-6	F13G11.1d	Transcription factor Doublesex [KOG3815]	?	CaMK1d

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
dpy-31	R151.5	an astacin zinc-metalloprotease of the BMP-1 (bone morphogenetic protein-1)/TOLLOID family	Yes	CaMK1g
epac-1	T20G5.5	Exchange Protein Activated by Cyclic AMP	?	CaMK1b
epac-1	T20G5.5	Exchange Protein Activated by Cyclic AMP	?	CaMK4
fbxa-9	Y54F10BM.5	F-box A protein	?	CaMK1a, 1b, 1d
fbxb-38	C33C12.5	protein containing an F-box	?	CaMK1a, 1d
fbxc-8	F58H7.7	a protein containing an F-box	?	CaMK1a, 1d
frm-1	ZK270.2	FERM domain (protein4.1-ezrin-radixin-moesin) family	?	CaMK4
fshr-1	C50H2.1	a putative neuropeptide receptor required for normal acetylcholine secretion by synapses	Yes	CaMK4
gcy-31	T07D1.1	a soluble guanylyl cyclase	Yes	CaMK1g
glb-29	Y17G7B.6	encodes a globin	Yes	CaMK1g
glb-8	C26C6.7	glb-8 encodes a globin	No	CaMK1b
glt-6	R05G6.6	glt-6 encodes an ortholog of glutamate/aspartate and neutral amino acid transporters	No	CaMK1b
hda-4	C10E2.3	a class II histone deacetylase	Yes	CaMK1g
hpl-1	K08H2.6	heterochromatin protein 1 (HP1) homologs	Yes	CaMK4
inx-11	W04D2.3	a predicted member of the innexin family.	Yes	CaMK1g
itx-1	W03D8.6	Caspr orthologues (belong to the Neurexin superfamily)	No	CaMK4
larp-1	R144.7	protein containing an La RNA-binding motif and a conserved LARP1 domain	?	CaMK1a, 1d
lge-1	K09C8.4		?	CaMK1b
math-29	F45C12.10	Uncharacterized protein, contains BTB/POZ domain	?	CaMK1b
mel-11	C06C3.1	ortholog of the vertebrate smooth muscle myosin-associated phosphatase regulatory subunit (PP-1M)	No	CaMK1g
mms-19	C24G6.3	DNA repair/transcription protein Mms19	?	CaMK1a, 1d
mnp-1	B0285.7	a protein related to the M1 family of metalloproteinases	No	CaMK1g
mtm-5	H28G03.6	a large (1,744-residue) putative SET-binding factor orthologous to human SBF1 (OMIM:603560) and SBF2 (OMIM:607697, mutated in Charcot-Marie-Tooth disease, type 4B2)	No	CaMK1g
mut-16	B0379.3	a glutamine/asparagine (Q/N)-rich domain-containing protein that lacks obvious homologs outside of other nematode species	?	CaMK4
nas-17	K03B8.2	encodes an astacin-like metalloprotease	?	CaMK1b
nas-29	F58A6.4	Nematode AStacin protease)	?	CaMK1a, 1d
nca-2	C27F2.2	nca-2 encodes a novel, four-domain alpha1U Ca2+ channel subunit	Yes	CaMK1b
ndg-4	F56F3.2	Predicted acyltransferase	No	CaMK4
nhr-263	F10G2.9	Nuclear Hormone Receptor family	?	CaMK1g

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
nhr-275	Y5H2A.2	Nuclear Hormone Receptor family	?	CaMK1g
nhr-284	T20C7.2	Nuclear Hormone Receptor family	?	CaMK1g
nhr-71	K11E4.5	Nuclear Hormone Receptor family	No	CaMK1g
npp-8	Y41D4B.19	Nuclear pore complex, Nup155 component	?	CaMK1a, 1b, 1d
nsh-1	F20H11.2	Notch Signaling pathway Homolog	?	CaMK1g
oac-3	C06B3.2		?	CaMK1a
oct-1	F52F12.1	induces the transport of the prototypical organic cation tetraethylammonium.	Yes	CaMK1b
ogt-1	K04G7.3	O-linked N-acetylglucosamine (O-GlcNAc) transferase	Yes	CaMK1?
pam-1	F49E8.3	pam-1 encodes a metalloprotease that is the C. elegans puromycin-sensitive aminopeptidase (PSA) ortholog;	Yes	CaMK1b
pde-1	T04D3.3	Cyclic GMP phosphodiesterase	Yes	CaMK1a, 1d
pde-4	R153.1	a cAMP phosphodiesterase orthologous to Drosophila dunce and the mammalian PDE4 family of phosphodiesterases	?	CaMK1b
pes-7	F09C3.1	Ras GTPase-activating protein family	Yes	CaMK1a, 1b, 1d
pix-1	K11E4.4	PIX (PAK (p21-activated kinase) Interacting eXchange factor) homolog	?	CaMK1g
pkn-1	F46F6.2	Serine/threonine protein kinase	Yes	CaMK1a, 1b, 1d
plst-1	Y104H12BL.1	PLaSTin (actin bundling protein) homolog	?	CaMK4
pmk-2	F42G8.3	homolog of p38, a type of mitogen-activated protein kinase (MAPK)	?	CaMK1b
pqn-67	T16G1.1	predicted to contain a glutamine/asparagine (Q/N)-rich ('prion') domain	?	CaMK4
pqn-80	Y111B2A.14b	The protein product of this gene is predicted to contain a glutamine/asparagine (Q/N)-rich ('prion') domain	?	CaMK1b
pyk-1	F25H5.3e	pyk-1 encodes one of two C. elegans pyruvate kinases	?	CaMK1b
ret-1	W06A7.3		No	CaMK1b
rnp-5	K02F3.11	a putative member of the exon-exon junction complex, orthologous to human RNPS1 (OMIM:606447)	?	CaMK4
rsp-4	EEED8.7	Predicted splicing factor, SR protein superfamily	Yes	CaMK4
rsp-7	D2089.1	Splicing factor, arginine/serine-rich	?	CaMK4
rsp-8	C18D11.4	SR Protein (splicing factor)	Yes	CaMK4
rsr-1	F28D9.1	Splicing coactivator SRm160/300, subunit SRm160	?	CaMK4
rsr-2	Y57A10A.19	Splicing coactivator SRm160/300, subunit SRm300	?	CaMK4
sdc-2	C35C5.1	a protein that represses transcription of X chromosomes to achieve dosage compensation and to elicit hermaphrodite differentiation	?	CaMK1g
sec-15	C28G1.3		?	CaMK1d
seu-1	Y73B6BL.5	encodes proteins required for completely normal signalling by UNC-6/netrin and its UNC-5	?	CaMK4

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
		receptor		
srbc-40	H24O09.1		?	CaMK1a, 1b, 1d
sre-21	C47A10.4	Sre G protein-coupled chemoreceptor	Yes	CaMK1a, 1d
sre-9	C17E7.3	Serpentine Receptor, class E (epsilon)	?	CaMK1g
srg-28	T09D3.2	Receptor-like protein, Srg family	?	CaMK1a, 1d
srg-41	T19C4.3	Receptor-like protein, Srg family	?	CaMK1a, 1d
sri-46	K07E8.11	Predicted olfactory G-protein coupled receptor	?	CaMK1a, 1d
sri-48	ZC239.9	Predicted olfactory G-protein coupled receptor	?	CaMK1a, 1b, 1d
srj-18	R05D8.2	7-transmembrane olfactory receptor	?	CaMK1a, 1d
srsx-17	F58D7.1		?	CaMK1a, 1d
srsx-17	F58D7.1		?	CaMK1g
srsx-33	T19B10.10	Serpentine Receptor, class SX	?	CaMK1g
sru-19	T04A11.8		?	CaMK1a, 1d
srv-2	C14A4.15	Uncharacterized protein with weak similarity to 7-transmembrane receptor	?	CaMK1a, 1d
srw-2	T03D3.3	7-transmembrane olfactory receptor	?	CaMK1a, 1d
stn-2	F27D9.8	a gamma-syntrophin	?	CaMK1g
str-93	F59A1.14	Seven TM Receptor	?	CaMK1g
syp-2	C24G6.1	coiled-coil protein that functions during meiosis	?	CaMK1b
taf-2	Y37E11B.4	taf-2 encodes a member of the peptidase M1 family, a predicted aminopeptidase with similarity to human TBP-associated factor 2.	?	CaMK1d
tag-163	M01E11.7	Focal adhesion protein Tensin, contains PTB domain	No	CaMK1b
tag-325	C38D4.5	Predicted Rho GTPase-activating protein	?	CaMK4
tag-342	B0464.8		?	CaMK1a, 1b, 1d
tdc-1	K01C8.3	Aromatic-L-amino-acid/L-histidine decarboxylase	Yes	CaMK1g
tmd-2	C08D8.2	Tropomodulin and leiomodulin	?	CaMK1g
toh-1	T24A11.3	toh-1 encodes an astacin-like metalloprotease; TOH-1 is predicted to function as a secreted protease.	?	CaMK1a, 1d
ttn-1	W06H8.8	orthologs of titin (OMIM:188840, mutated in cardiomyopathy or tibial muscular dystrophy)	No	CaMK1g
ubh-4	C08B11.7		?	CaMK1d
ugt-33	C35A5.2	UDP-glucuronosyl and UDP-glucosyl transferase	?	CaMK1g
ugt-35	C32C4.7	UDP-Glucuronosyl Transferase	?	CaMK1g
ugt-36		UDP-Glucuronosyl Transferase	?	CaMK1g
ugt-37	F10D2.6	UDP-glucuronosyl and UDP-glucosyl transferase	?	CaMK1g
unc-129	C53D6.2	member of TGF-beta family of secreted growth	Yes	CaMK1a,

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
		factor signaling molecules		1d
unc-16	ZK1098.10	a homolog of murine JIP3/JSAP and Drosophila SUNDAY DRIVER; unc-16 is involved in vesicle transport and mutations affect egg laying, locomotion, and defecation	Yes	CaMK1g
unc-68	K11C4.5	ryanodine receptor ortholog	No	CaMK1a, 1b, 1d
unc-68	K11C4.5b	ryanodine receptor ortholog	No	CaMK1d
unc-80	F25C8.3d	uncharacterized	Yes	CaMK1b
unc-89	C09D1.1	required for proper organization of A bands in striated muscle	No	CaMK1b
vha-7	C26H9A.1	an ortholog of subunit a of the membrane-bound (V0) domain of vacuolar proton-translocating ATPase (V-ATPase)	No	CaMK1g
wrk-1	F41D9.3	a GPI-anchored immunoglobulin superfamily (IgSF) protein orthologous to Drosophila KLINGON, WRAPPER, CG7166, and CG13506	Yes	CaMK1g
wve-1	R06C1.3	a homolog of the mammalian WAVE protein that may be involved in actin cytoskeletal dynamics	Yes	CaMK1g
ztf-18	T24C4.7	Zinc finger Transcription Factor family	Yes	CaMK4
zyg-12	ZK546.1	Hook protein family members: act as linker proteins that connect membrane compartments with the microtubule cytoskeleton	Yes	CaMK1a, 1d
	B0001.8		?	CaMK4
	B0207.7	Casein kinase (serine/threonine/tyrosine protein kinase)	?	CaMK4
	B0336.11		?	CaMK1b
	C04G6.6		?	CaMK4
	C14A4.13	Casein kinase (serine/threonine/tyrosine protein kinase)	?	CaMK4
	C17H11.1	7 transmembrane receptor	?	CaMK4
	C18B12.2	a G-protein-coupled receptor (GPCR) that is a member of the secretin family (also known as family B or family 2) of GPCRs	?	CaMK1g
	C18H2.1		?	CaMK4
	C26E6.12	Predicted GTP-binding protein (ODN superfamily)	?	CaMK1b
	C28G1.2		?	CaMK4
	C29A12.6		?	CaMK1a, 1d
	C34H4.5		?	CaMK4
	C36B1.11		?	CaMK4
	C42D4.1	Predicted alpha-helical protein	?	CaMK1a, 1d
	C42D8.9		?	CaMK1b
	C44B9.3		?	CaMK1a, 1d
	C44C10.4	Structural maintenance of chromosome protein 1 (sister chromatid cohesion complex Cohesin, subunit SMC1)	?	CaMK1a, 1d
	C45G3.5	Lipoate-protein ligase A	?	CaMK1a, 1d

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
	C46E10.3		?	CaMK1a, 1d
	C47E12.11		?	CaMK1g
	D1037.1	DNA binding protein SON	?	CaMK4
	D1086.12		?	CaMK1g
	D2092.6		?	CaMK1g
	F01G4.4		?	CaMK1g
	F08F8.10		?	CaMK1a, 1d
	F08G2.8		?	CaMK1a, 1d
	F10D7.5	ortholog of Drosophila NEURALIZED, and thus may participate in GLP-1/LIN-12 signalling	?	CaMK1b
	F13H6.1	Zn-finger	?	CaMK4
	F13H6.1		?	CaMK4
	F14F7.4		?	CaMK1b
	F16B12.7	Casein kinase (serine/threonine/tyrosine protein kinase)	?	CaMK1b
	F22B3.9		?	CaMK1b
	F26F2.1		?	CaMK1b
	F28C1.1	SWAP mRNA splicing regulator	?	CaMK4
	F33H2.5	DNA polymerase epsilon, catalytic subunit A	No	CaMK4
	F34D10.9		?	CaMK1b
	F39B2.7	mitochondial GTPase	?	CaMK1a
	F39B2.7	Mitochondrial GTPase	?	CaMK1a, 1d
	F40A3.5		?	CaMK1g
	F40F12.4		?	CaMK1a, 1b, 1d
	F40F12.4		?	CaMK1a, 1d
	F41E6.12		?	CaMK1g
	F44A2.3		No	CaMK1g
	F45E4.11	Permease of the major facilitator superfamily	?	CaMK1a, 1d
	F46C5.7		?	CaMK1a, 1d
	F47G4.4	F47G4.4 encodes a paralog of MEI-2 that, like MEI-2, binds MEI-1 in vitro; F47G4.4 might thus be an alternative ligand of MEI-1 in vivo.	?	CaMK4
	F48C1.6		?	CaMK4
	F49C12.15		?	CaMK4
	F52C9.7		?	CaMK4
	F52E1.13	Oxidation resistance protein	Yes	CaMK1b
	F54B3.1		?	CaMK1b
	F54B3.1		?	CaMK1g
	F54C8.4	mRNA capping enzyme, guanylyltransferase (alpha) subunit	No	CaMK1g

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
	F55B11.6		?	CaMK1g
	F56A11.6		?	CaMK4
	F56C3.5		?	CaMK1a, 1d
	F56D2.6	a DEAH helicase orthologous to the Drosophila CG11107, the human DDX15, and the S. cerevisiae PRP43 proteins.	?	CaMK4
	F59A3.12		?	CaMK1g
	H23L24.4		?	CaMK1b
	H35B03.1		?	CaMK1a, 1b, 1d
	H35N09.2		?	CaMK1a, 1d
	K02F6.3	WSN domain	?	CaMK1g
	K02F6.4	WSN domain	?	CaMK1g
	K03A11.4		?	CaMK1a, 1d
	K04H4.2	predicted to be secreted, with an N-terminal chitin-binding peritrophin-A domain followed by up to 15 cysteine-rich domains.	?	CaMK1b
	K05C4.11	Cubilin, multiligand receptor mediating cobalamin absorption	?	CaMK1g
	K05C4.5		?	CaMK1a, 1b, 1d
	K07D4.4		?	CaMK1a, 1d
	K10B3.6	Predicted starch-binding protein	?	CaMK1b
	K12C11.6		?	CaMK1g
	M02B7.5		Yes	CaMK1b
	R01H10.7	Inositol-polyphosphate 4-phosphatase	?	CaMK1a, 1d
	R03E1.4		Yes	CaMK1a
	R05F9.11	Small nuclear RNA activating complex (SNAPc), subunit SNAP43	?	CaMK1b
	R07B7.2		?	CaMK1b
	R08C7.12		?	CaMK1a, 1d
	R09A8.1		?	CaMK1b
	R10E11.6	previously undescribed V-ATPase component or ancillary protein; specifically	?	CaMK1a, 1d
	R160.6		?	CaMK1g
	R31.2		?	CaMK1b
	T01B6.3	T01B6.3 encodes one of five C. elegans AMP kinase (AMPK) gamma regulatory subunits	?	CaMK1b
	T02G5.11		?	CaMK1g
	T04D1.2	protein containing an F-box	?	CaMK1a, 1d, 1g
	T05H4.15		?	CaMK1g
	T06A10.3		?	CaMK1g
	T07D10.1		?	CaMK4

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
	T09E11.12		?	CaMK1a, 1d
	T09F3.5		?	CaMK1a, 1b, 1d
	T10B5.10		?	CaMK1a, 1d
	T12E12.6	Puromycin-sensitive aminopeptidase and related aminopeptidases	?	CaMK1g
	T16G12.6	Nuclear transport receptor LGL2 (importin beta superfamily)	?	CaMK1a, 1d
	T21D11.1	Gelatinase A and related matrix metalloproteases	?	CaMK1a, 1d
	T21E8.7		?	CaMK1b
	T22B2.3		?	CaMK1a, 1d
	T23F1.5	Cuticulin precursor	?	CaMK1a, 1d
	T24A6.7	Predicted riboflavin biosynthesis protein	?	CaMK1g
	T25B2.2		?	CaMK1g
	T26A5.5	F-box protein JEMMA and related proteins with JmjC, PHD, F-box and LRR domains	Yes	CaMK1a, 1b, 1d
	T26A8.4	ortholog of Saccharomyces cerevisiae Caf120 (regulates transcription and RNA degradation)	?	CaMK4
	T27E9.6		?	CaMK4
	T27F6.10		?	CaMK1a, 1d
	T28F4.4		?	CaMK1g
	W02D7.8		?	CaMK4
	W04A8.1	weakly similar to human microcephalin	?	CaMK1a, 1b, 1d
	W06D11.5		?	CaMK1a, 1d
	Y105E8A.28		?	CaMK1g
	Y15E3A.3		?	CaMK1g
	Y16B4A.2		?	CaMK1b
	Y19D2B.1	Alpha tubulin	?	CaMK1a, 1d
	Y25C1A.8	Conserved Zn-finger protein	?	CaMK4
	Y39A3CL.7		?	CaMK4
	Y39A3CR.3		No	CaMK1g
	Y43F8B.16		?	CaMK1b
	Y43F8B.1d		?	CaMK1b
	Y45F10B.15		?	CaMK1a, 1d
	Y47D7A.14		?	CaMK1a, 1d
	Y47H9C.9		?	CaMK1g
	Y48C3A.12		?	CaMK1g
	Y48C3A.5		?	CaMK1b
	Y51H1A.1	Uncharacterized conserved protein, contains C1,	?	CaMK4

Gene name	Sequence Name	Description	Neuronal Expression?	Predicted target of:
		PH and RUN domains		
	Y54F10BM.9		?	CaMK4
	Y55F3AM.3	Transcriptional coactivator CAPER (RRM superfamily)	?	CaMK4
	Y59A8B.21		?	CaMK1a, 1d
	Y66D12A.19		?	CaMK1b
	Y6B3B.1		No	CaMK1g
	Y6D1A.1		?	CaMK1a, 1b, 1d
	Y71F9AR.2		?	CaMK1g
	Y71F9B.6		?	CaMK1b
	ZC247.1		?	CaMK4
	ZK287.7		?	CaMK1g
	ZK337.2	Zn-finger	No	CaMK1g
	ZK792.1	Histidine acid phosphatase	Yes	CaMK1a, 1d

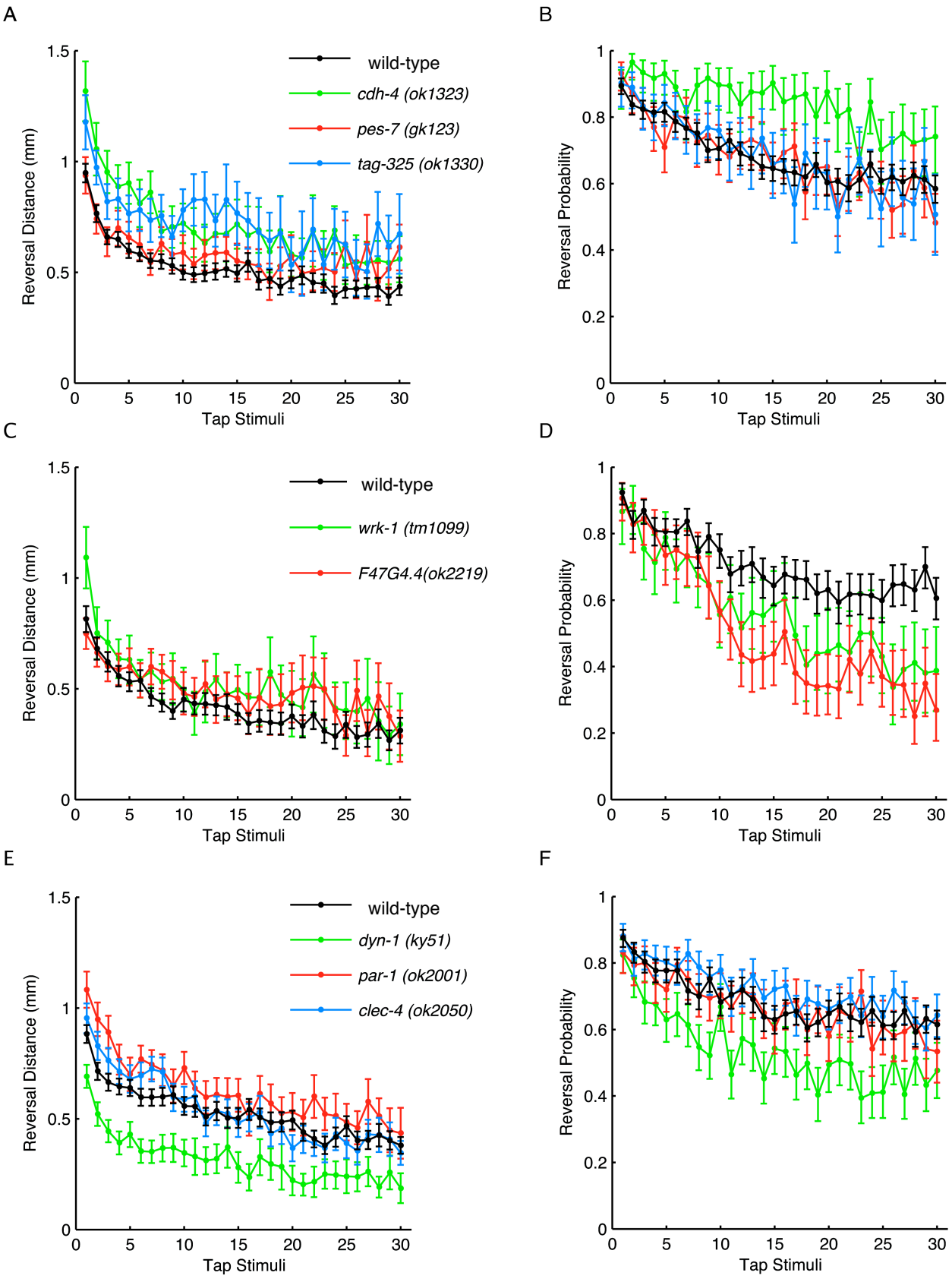

Figure 3.6 Habituation curves of 96-hour old wild-type and mutant strains.

Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

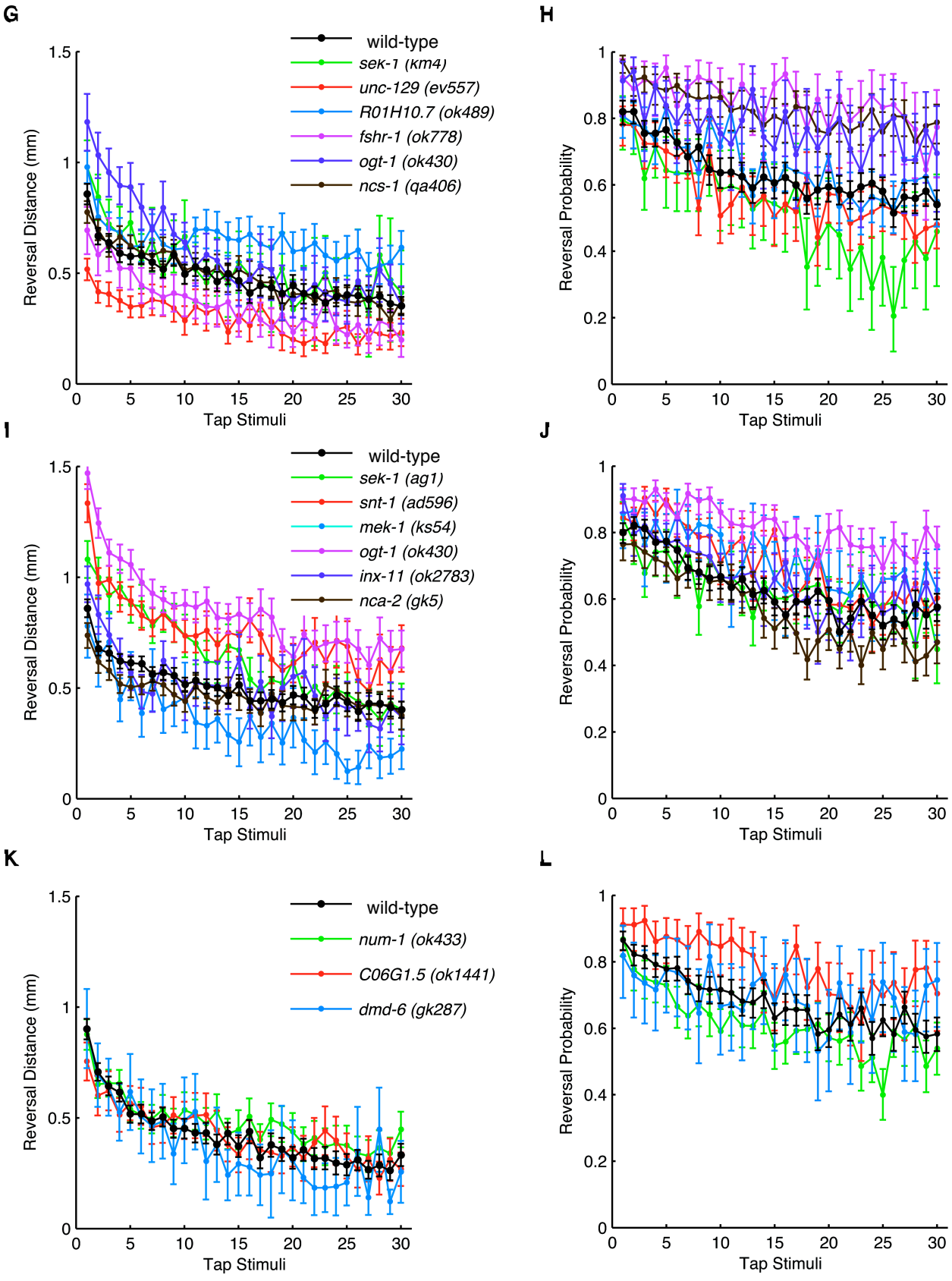

Figure 3.6 Habituation curves of 96-hour old wild-type and mutant strains.
Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

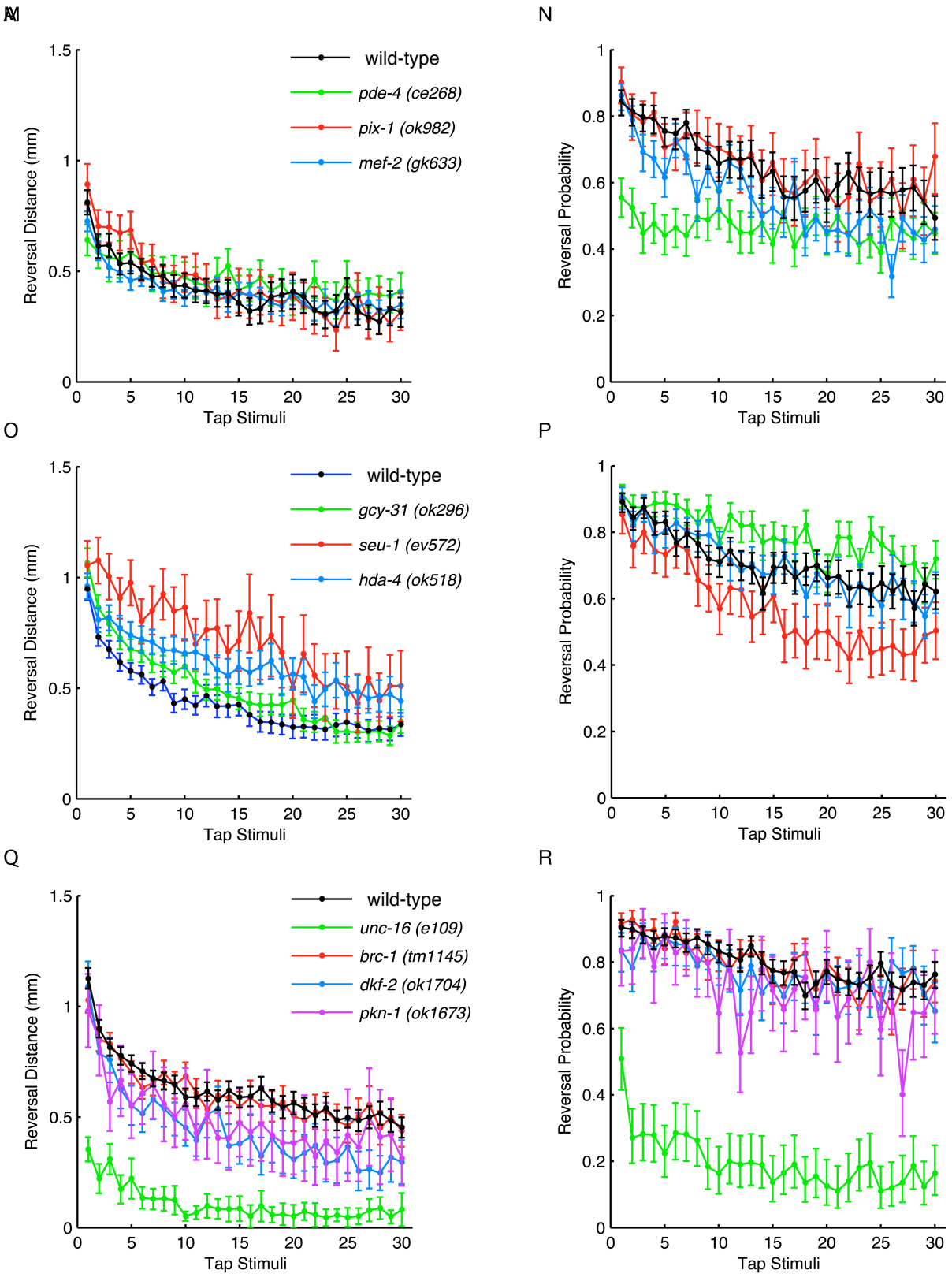

Figure 3.6 Habituation curves of 96-hour old wild-type and mutant strains.
Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

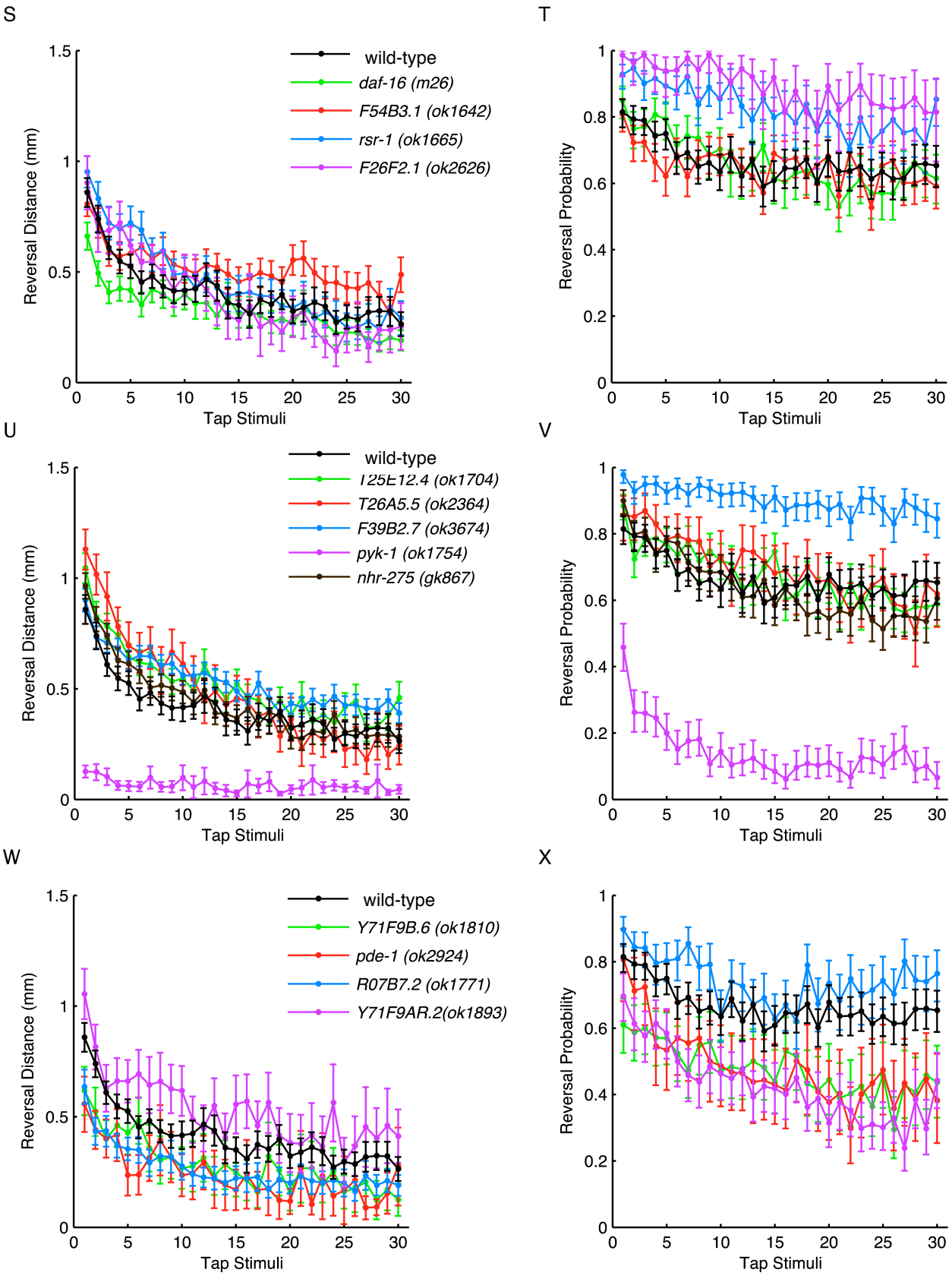

Figure 3.6 Habituation curves of 96-hour old wild-type and mutant strains.
Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

Table 3.3 Statistical results from habituation screen of putative CaMK1 phosphorylation targets

genotype	Reversal Distance				Reversal Probability			
	Initial tap		Final tap		Initial tap		Final tap	
	p-value	score	p-value	score	p-value	score	p-value	score
brc-1(tm1145)	NS		NS		NS		NS	
C06G1.5(ok1441)	NS		NS		NS		NS	
cdh-1(ok1323)	<0.01	++	NS		NS		<0.05	+
clec-4(ok2050)	NS		NS		NS		NS	
daf-16(m26)	<0.05	-	NS		NS		NS	
dkf-2(ok1704)	NS		NS		NS		NS	
dmd-6(gk287)	NS		NS		NS		NS	
dyn-1(ky51)	NS		<0.01	--	NS		NS	
F26F2.1(ok2626)	NS		NS		<0.05	+	NS	
F39B2.7(ok3674)	NS		<0.05		<0.0001	++++	<0.01	++
F47G4.4(ok2219)	NS		NS		NS		<0.01	--
F54B3.1(ok1642)	NS		<0.001	+++	NS		NS	
fshr-1(ok778)	NS		NS		NS		NS	
gcy-31(ok296)	NS		NS		NS		<0.05	+
hda-1(ok518)	NS		NS		NS		NS	
inx-11(ok2783)	NS		NS		NS		NS	
mef-2(gk633)	NS		NS		NS		NS	
mek-1(ks54)	NS		NS		NS		NS	
nca-2(gk5)	NS		NS		NS		NS	
ncs-1(qa406)	NS		NS		<0.001	+++	0.001	+++
nhr-275(gk867)	NS		NS		<0.01	+++	NS	
num-1(ok433)	NS		NS		NS		NS	
ogt-1(ok430)	<0.05	+	NS		NS		NS	
ogt-1(ok1474)	<0.001	+++	<0.001	+++	NS		<0.01	++
par-1(ok2001)	<0.05	+	NS		NS		NS	
pde-1(ok2924)	<0.05	-	NS		NS		<0.01	--
pde-4(ce268)	NS		NS		<0.0001	----	NS	
pes-7(gk123)	NS		NS		NS		NS	
pix-1(ok982)	NS		NS		NS		<0.01	++
pkn-1(ok1673)	NS		NS		NS		NS	
pyk-1(ok1754)	<0.001	---	NS		<0.0001	----	<0.0001	----
R01H10.7(ok489)	NS		<0.001	+++	NS		NS	
R07B7.2(ok1771)	<0.01	--	NS		NS		NS	
rsr-1(ok1665)	NS		NS		<0.05	+	<0.01	++
sek-1(ag1)	NS		NS		NS		NS	
sek-1(km4)	NS		NS		NS		NS	
seu-1(ev572)	NS		NS		NS		<0.05	-
snt-1(ad596)	<0.001	+++	<0.001	+++	NS		NS	
T25E12.4(ok1704)	NS		<0.001		<0.05	+	NS	
T26A5.5(ok2364)	<0.05	+	NS		NS		NS	
tag-325(ok1330)	NS		<0.05	+	NS		NS	
unc-129(ev557)	<0.001	---	NS		NS		NS	
unc-16(e109)	<0.0001	----	NS		<0.0001	----	<0.0001	----
wrk-1(tm1099)	<0.01	++	NS		NS		<0.05	-
Y71F9AR.2(ok1893)	NS		NS		<0.05	-	<0.01	--
Y71F9B.6(ok1810)	<0.01	++	NS		<0.0001	----	<0.01	--

"+" represents a phenotype of greater responding than wild-type worms
"-" represents a phenotype of lesser responding than wild-type worms

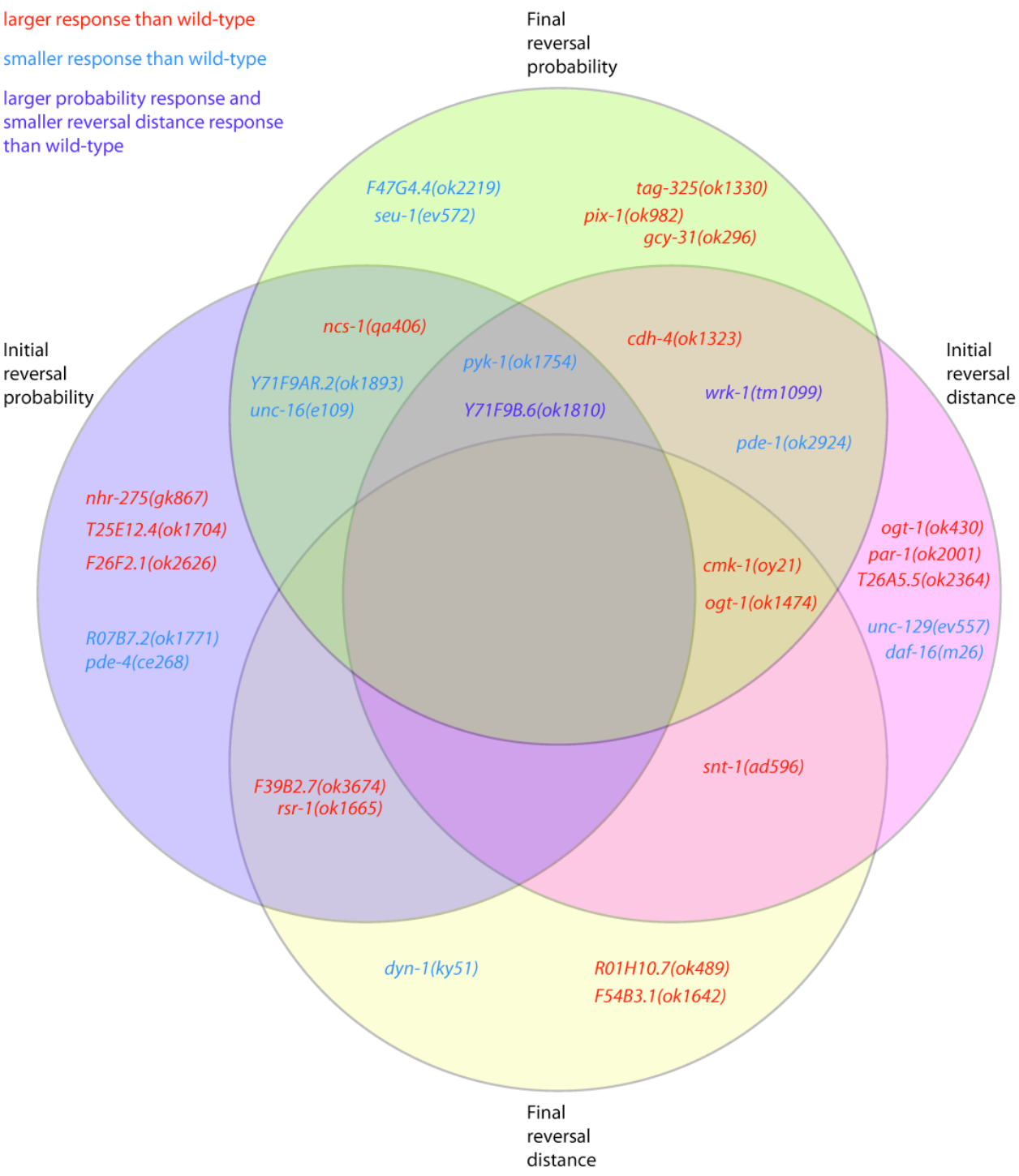

Figure 3.7 Summary of 60s ISI habituation phenotypes.

Venn diagram grouping genes whose mutant alleles showed similar behavioural phenotypes when given 30 taps at a 60s ISI. Grouping was based on the statistical analysis presented in table 3.2.

3.4 Discussion

In summary, the data suggests that CMK-1, and not CKK-1, plays a role in the tap withdrawal response and habituation to tap at a 60s ISI. Specifically, *cmk-1(oy21)* mutants have larger naïve response to tap than wild-type worms, and both *cmk-1* mutant strains, but not *ckk-1*, habituate more shallowly than wild-type worms in the measures of reversal distance and response probability. This data is supported by experiments demonstrating that only CMK-1, but not CKK-1, is expressed within the mechanosensory neurons and the interneurons of the tap withdrawal circuit. These habituation phenotypes are caused by mutations in *cmk-1* and not other background mutations as the habituation phenotype is observable in two strains with independently generated mutant alleles of *cmk-1* and because expressing a wild-type copy of the CMK-1 cDNA in *cmk-1* mutants rescues these habituation deficits.

Although previous studies suggested that CMK-1 and CKK-1 function in the same pathway our results indicating that CMK-1, but not CKK-1, is required for habituation are not the first findings to suggest that CaMK1 can function independently of CaMKK. In *C. elegans*, Kimura and colleagues (Kimura et al., 2002) found that although CKK-1 enhanced the CMK-1-dependent phosphorylation of a transcription factor (CREB), CMK-1 was able to phosphorylate CREB in the absence of CKK-1. In this study they also showed that in *C. elegans* CMK-1 is expressed in more neurons than is CKK-1. Similarly, Satterlee et al (2004) found that CMK-1, but not CKK-1, functioned to regulate AFD sensory neuron specific gene expression in *C. elegans*. These findings also appear to be consistent with studies on vertebrate organisms where CaMK1 is known to have a wider expression profile than does CaMKK (reviewed in Hook and Means, 2001). These data suggest that sometimes activation of CaMK1 by calmodulin alone may be sufficient to activate the kinase in the context of some biological signaling or that sometimes CaMK1 is activated via phosphorylation by another unidentified kinase. These hypotheses are not necessarily mutually exclusive.

At a 60s ISI, neither of the worm strains with mutant alleles of *cmk-1* habituated as deeply as wild-type worms for both behavioural measures, reversal distance and response probability. In contrast, when worms were habituated at a 10s ISI only one allele of *cmk-1* appeared to affect the final habituated level and that was only in the measure of reversal distance. The robust habituation phenotype of *cmk-1* mutants at a 60s ISI and very modest phenotype at a 10s ISI supports the hypothesis that habituation at different ISIs is mediated by different molecular mechanisms (Broster and Rankin, 1994).

One potential confound with interpreting the phenotype of larger reversal distance of habituated level in *cmk-1* mutants as a learning defect is the possibility that this phenotype occurs because *cmk-1* mutants simply give larger reversals in response to all taps; *cmk-1* mutants did give larger reversals in response to the initial tap. Two pieces of evidence suggest that this is not the case: 1) *cmk-1* mutants can habituate as deeply as wild-type mutants (i.e. respond to tap with very small reversals) if they are habituated at a 10s ISI, despite the increased reversal distance phenotype to the initial tap and 2) younger, 72 hour-old worms, *cmk-1* mutants do not respond to the initial tap with a larger reversal distance than wild-type worms but the learning phenotype (not habituating as deeply as wild-type at a 60s ISI) is still present. Thus these data strongly suggest that the phenotype of larger reversal distance of habituated level in *cmk-1* mutants is a learning defect.

The behavioural screen of potential downstream targets of CMK-1 yielded 4 strains with mutations in *cdh-4, snt-1, ogt-1,* and *F39B2.7* that phenocopied *cmk-1* mutant worms in at least 2 behavioural measures.

cdh-4 is the *C. elegans* Fat-like cadherin homologue (Schmitz et al., 2008). Fat-like cadherins are members of the cadherin superfamily and are characterized by a large number of cadherin module repeats in their extracellular domain (Tanoue and Takeichi, 2005). Previously Fat-like cadherins have been shown to function in regulating cell proliferation (Drosophila;

Bryant et al., 1988), cell polarity (Drosophila; Yang et al., 2002; Matakatsu and Blair, 2004), and cell–cell interactions in the kidney and brain that are necessary for development (mice; Ciani et al., 2003).

In *C. elegans cdh-4* has recently been shown to function in many aspects of embryonic development, specifically: fasciculation of the major longitudinal axon tracts, neuroblast migration, pharynx development and hypodermal development (Schmitz et al., 2008). It may be that the observed behavioural phenotypes in this study are due to CDH-4's role in embryonic development of the nervous system (i.e. *cdh-4* mutants may have a differently wired nervous system than do wild-type animals) and not its role in adult learning. Schmitz and colleagues (2008) observed that *cdh-4* mutants exhibited fasciculation defects of *glr-1* expressing interneurons in the ventral nerve cord: in contrast to wild-type animals, in *cdh-4* mutants these interneurons crossed frequently between the left and right axon tracts. Schmitz and colleagues (2008) also found that the motorneurons also showed fasciculation defects. The motorneurosn AVA L/R, AVD L/R, AVB L/R and PVC L/R (*glr-1* expressing interneurons) are a large proportion of the neurons that make up the tap withdrawal circuit (Wicks and Rankin, 1995) and alteration in axonal fasciculation of these neurons could cause synaptic miswiring that may explain some of the observed habituation defects in these mutants. Other defects observed by Schmitz and colleagues (2008) include the Q neuroblast migration. In wild-type animals the right Q neuroblast (QR) and its descendants, SDQR and AVM, migrate anteriorly. In a small proportion of *cdh-4* mutants it was observed that these cells ended up in the posterior of the worm instead. Since AVM is required for worms to reliably respond to tap (Wicks and Rankin, 1995) this alteration could potentially cause this small proportion of the population to sense tap abnormally and could also contribute the behavioural phenotype I observed. In wild-type animals the left Q neuroblast (QL) and its descendants, SDQL and PVM, migrate posteriorly. In about half of the *cdh-4* mutant worms these cells were found to migrate anteriorly. Although this

affects a larger proportion of *cdh-4* mutants than the QR migration defect it is less likely to affect tap habituation as PVM is not thought to function in the tap withdrawal response (ablation of PVM does not alter the tap withdrawal response or habitation; Wicks and Rankin, 1995). Because the tests of learning and memory used in this study use a locomotory behaviour as an output to measure whether or not worms have learned or remembered any gene that alters locomotion (such as by altering the wiring of the locomotory circuit) could appear to affect memory even if it does not directly function that way. Thus these behavioural data from *cdh-4* mutants may not help us understand adult learning but may be very useful in the context of trying to understanding how alterations in neuronal wiring affects behaviour. Although, it is important to note that the allele tested in the present study *cdh-4(ok1323)* appears to be a large deletion in the protein, suggesting it is likely a null mutation, it did not display the same fasciculation of the major longitudinal axon tracts, neuroblast migration, pharynx development and hypodermal development defects observed in other *cdh-4* alleles. Backcrossing the *cdh-4(ok1323)* allele into the wild-type strain and rescue experiments with a wild-type copy of *cdh-4* in this strain may help clarify if it is the identified mutation in *cdh-4* in this strain that is responsible for the observed habituation defects, regardless of whether they are due to the protein's role in development or adult learning.

snt-1 is the *C. elegans* homologue of Synaptotagmin 1 (Nonet et al., 1993); an abundant integral membrane protein in synaptic vesicles (Matthew et al., 1981). Synaptotagmins are comprised of a single transmembrane domain and two Ca^{2+} binding domains (Perin et al., 1991). *In vitro* and *in vivo* studies have demonstrated that Synaptotagmin 1 is involved in vesicle fusion and endocytosis (Brose et al., 1992; Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Fernandez et al., 2001; Fernandez-Chacon et al., 2001; Pang et al., 2006). It is hypothesized to function as a Ca^{2+} sensor for the regulated exocytosis of neurotransmitters by preventing constitutive neurotransmitter vesicle fusion until Ca^{2+} entry. Knocking out Synaptotagmin 1 in

Drosophila, *C. elegans* and mice results in dramatically impaired synaptic function, but in all of these cases there appears to be some amount of synaptic activity left (DiAntonio et al., 1993; Littleton et al., 1993; Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994).

There is some evidence that Synaptotagmin 1 may play a role in skill learning in rats (Akita et al., 2006) or modulation of learning by exercise in rats (Liu et al., 2009) but because disruption of Synaptotagmin 1 results in a large reduction of neurotransmitter release it is hard to dissect whether or not Synaptotagmin 1 plays a modulatory role in learning or whether synaptic vesicle release is what is critical, and that disrupting any form of it would result in the same behavioural outcome. But, even if the latter is the case, this may be very informative about habituation of the tap withdrawal response: *snt-1* mutants have decreased synaptic vesicles (Jorgensen et al., 1995), as well as synaptic transmission (Nonet et al., 1993; Nguyen et al., 1995; Miller et al., 1996) but are as likely to respond to tap as wild-type animals and respond to tap with larger reversal distances. This suggests that chemical synaptic transmission may not be critical for the response to tap, and that reversal size may be negatively modulated by synaptic vesicle release. These mutants also do not habituate as deeply as wild-type animals in the measure of reversal distance and this may suggest that reversal distance habituation may be mediated by a potentiation of synaptic vesicle release that acts to decrease reversal distance further.

The *C. elegans* gene *ogt-1* is an ortholog of O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT; Lubas et al., 1997). O-GlcNAc glycosylation is a unique and dynamic cytosolic and nuclear carbohydrate post-translational modification in which β-N-acetylglucosamine is covalently attached to serine or threonine residues of proteins. In contrast to other forms of glycosylation, O-GlcNAc glycosylation occurs intracellularly and is not further modified into complex glycans. It is also a rapid posttranslational modification that has been observed to occur as quickly as 1-5 min after cellular stimulation (Golks et al., 2007; Song et al.,

2008). Because of these properties, this post-translational modification it is thought to be more akin to phosphorylation than to other forms of glycosylation. Interestingly, O-GlcNAc glycosylation sometimes occurs on or near serine or threonine residues that are also known to undergo phosphorylation (reviewed in Rexach et al., 2008). Both OGT and O-GlcNAcase (enzyme that functions in opposition to O-GlcNAc transferase to remove β-N-acetylglucosamine residues; OGA) are highly expressed in the brain (Kreppel et al., 1997; Gao et al., 2001) and enriched at synapses within neurons (Cole and Hart, 2001; Akimoto et al., 2003).

Previously it has been shown that there is cross talk between O-GlcNAc glycosylation and CaMK signaling. Song et al (2008) demonstrated that in neuronal NG-108-15 cells depolarized by KCl OGT was phosphorylated by CaMK4 and that this stimulation increased the O-GlcNAc glycosylation of proteins in these cells in a CaMK4-dependent manner. Interestingly, another study showed that O-GlcNAc glycosylation could regulate CaMK4 function: Dias and colleagues (2009) showed that CaMK4 is highly GlcNAc glycosylated *in vivo* and that in response to ionomycin treatment (which increases Ca^{2+} signaling) CaMK4 increased its interaction with OGA leading to a decrease in the GlcNAc glycosylation and a decrease in its phosphorylation of Thr-200 (a phosphorylation site known to increase the catalytic activity of CaMK1 and CaMK4) of CaMK4. These two studies suggest a complex interaction between these proteins and may indicate a feedback mechanism to regulate CaMK signaling.

In *C. elegans,* although OGT-1 is expressed in the nervous system no role has yet been described for this gene in learning. Instead studies have focused on other biological processes and shown that it functions in macronutrient storage (Hanover et al., 2005), dauer formation (Lee et al., 2010), lifespan (Rahman et al., 2010), and the glucose stress response (Mondoux et al., 2011).

In other species the role of O-GlcNAc glycosylation in learning and memory has not been studied directly but it has been shown to function in cellular models of learning. O-GlcNAc

glycosylation of AMPA receptors (specifically GluR1 and GluR2) has shown to be critical (Din et al., 2010) for both LTP and LTD. This mechanism is unlikely to be conserved to short-term habituation in *C. elegans* because worms with mutations in the homologs of these genes, glr-1 and glr-2 show normal habituation at a 10 and 60 s ISI (Rose et al., 2003, and unpublished data from our lab), suggesting AMPA receptors are not necessary for short-term habituation.

O-GlcNAc glycosylation has also been implicated in another cellular model of learning, paired-pulse facilitation (Tallent et al., 2009). Paired pulse facilitation is a form of short-term plasticity that is dependent upon presynaptic mechanisms, specifically when a presynaptic neuron receives two stimuli within a very short time period, the postsynaptic response will usually be bigger for the second stimuli than it was for the first. Tallent and colleagues (2009) applied an inhibitor of OGA to increase the amount of O-GlcNAc glycosylation and observed that this manipulation decreased paired-pulse facilitation. They also showed that the application of this OGA inhibitor increased phosphorylation of the extracellular-signal-regulated kinase (Erk) and Synapsin (a protein that in a phospho-dependent manner binds synaptic vesicles to components of the cytoskeleton to prevent them from migrating from the reserve pool of vessicles to the ready releasable pool of vesicles located at the presynaptic membrane where they could release neurotransmitter). Erk is known to phosphorylate Synapsin to promote migration of vesicles from the reserve to the ready releasable pool (Kushner et al., 2005). Thus Tallent and colleagues (2009) propose a model where cellular depolarization leads to increases in intracellular Ca^{2+} concentrations that activate CaMK4. Once activated, CaMK4 phosphorylates and activates OGT (whose activation may in turn decrease CaMK4's activity via OGA), which leads to Erk's phosphorylation. Activated Erk can then phosphorylate Synapsin to facilitate the migration of synaptic vesicles from the reserve to the ready releasable pool causing an increase of neurotransmitter release during the second stimulus during paired pulse facilitation.

A mechanism similar to this may function during short-term tap habituation in *C. elegans*. But this sounds like a mechanism for synaptic facilitation – how could this result in the behavioural decrement observed in habituation? If the synapse this was occurring at was at an inhibitory synapse that acted to alter the flow of excitation within the tap withdrawal circuit then this mechanism could easily lead to the behavioural decrement seen during tap habituation. Potential synapses within the tap withdrawal circuit where this might occur are the putatively inhibitory chemical synapse between the mechanosensory neurons and the command interneurons (i.e. synapses from PLM to AVA and AVD and the synapses from ALM and AVM to AVB and PVC).

The *C. elegans* gene F39B2.7 is homologous to mammalian GTPBP3. It is an evolutionarily conserved protein that functions in mitochondrial tRNA modification. Disruption of human GTPBP3 by small interfering RNA resulted in decreased O_2 consumption, ATP production and mitochondrial protein synthesis (Villarroya et al., 2008). No role has been demonstrated for this protein in learning or memory but there is much evidence that supports mitochondrial function as being critical for this process: synaptic transmission caused prolonged activation of mitochondrial membrane conductance (Jonas et al., 1999), normal mitochondrial transport and distribution was required for normal synaptic transmission (Stowers et al., 2002; Guo et al., 2005; Verstreken et al., 2005), recruitment and mobilization of the reserve pool of vesicles at the presynapse was dependent on mitochondrial function (Verstreken et al., 2005), disruption of porin (a building block of the mitochondrial permeability transition pore) disrupted both fear conditioning and spatial learning in mice (Weeber et al., 2002), mitochondria-encoded genes were selectively up regulated after learning (Pinter et al., 2005), and tetanic stimulation triggered a fast delivery of mitochondria to the synapse that was necessary for synaptic potentiation (Tong, 2007). It could be that putative phosphorylation of *F39B2.7*/GTPBP3 by CMK-1 during short-term habituation could lead to increased *F39B2.7*/GTPBP3 activity,

resulting in increased ATP production that could be used to sustain prolonged synaptic transmission and presynaptic recruitment and mobilization of the reserve pool of vesicles.

Further experiments must be done to confirm that the identified mutations in these strains are responsible for the observed behavioural phenotypes and to confirm that they function in the same biochemical pathway as CMK-1 and each other. To test whether the identified mutation in these strains or other background mutations in the strains are the cause of the behavioural phenotypes one or more of the following approaches must be done: i) replicating the habituation phenotypes in strains with an independently generated mutant alleles of the same gene, ii) genetic rescue experiments, and/or iii) RNAi knockdown of the gene of interest. Second, I must test whether these genes act in the same biochemical pathway as CMK-1 by creating double mutant strains that have mutations in both *cmk-1* and the gene of interest. If these double mutant strains do not show an additive phenotype (either a larger reversal distance or a higher likelihood of responding to the final tap in a habituation series than either mutation causes alone) I can conclude that the gene of interest does function in the same biochemical pathway as CMK-1. Further research should also be directed at identifying where in the tap withdrawal circuit CMK-1 is functioning and determining if the putative downstream targets act at the same locus.

CHAPTER 4: Age-Related Changes in Habituation During Reproduction are the Result of Decreased Stimulus Intensity Discrimination

4.1 Introduction

Preliminary evidence from a 10s ISI habituation screen of mutant *C. elegans* strains suggested that the short-term habituation deficit observed in *cmk-1* mutants might be due to a delay in development or an acceleration of the ageing process (Giles et al., 2011). Further investigation of CMK-1 dismissed these concerns as *cmk-1* mutants habituated more slowly than wild-type worms at all ages tested but led to the observation that as wild-type worms age from 72 to 120 hrs-old their asymptotic level of habituation changed. This study was designed to examine this in greater detail.

We rely on learning and memory for most of our daily tasks, but our capacity to learn and remember changes dramatically during our lifespan and declines naturally with age. Although many studies have examined changes in learning and memory related to early development and changes related to old age, ageing-related changes during the early and middle adulthood period have been understudied (Alwin and Hofer, 2011). In studies of ageing, reproductive and middle-aged subjects are often pooled together and compared to geriatric cohorts potentially masking or diminishing any age-dependent changes within these groups. This common experimental approach may explain why there is no consensus on when cognitive decline begins and why most treatments are targeted to individuals in late adulthood (60-70 years of age; discussed in Salthouse, 2009). Understanding the changes in the mid-life years may lead to earlier interventions or treatments.

Age-dependent changes in learning and memory are not limited to humans and are conserved across the animal kingdom. Thus it is possible to use animal models to gain insight into the natural ageing process that results in cognitive decline, and to develop new approaches for treatment. However, large parametric studies of ageing and behaviour are almost impossible

to do in humans and mammalian model organisms due to difficulties and expenses in obtaining and maintaining a sufficient number of subjects of each age. In contrast, the model organism *Caenorhabditis elegans*, a nematode with a short-lifespan (reaches reproductive maturity in the lab at 3 days of age), makes an ideal model to study the relationship between ageing and learning (both associative and non-associative; Beck and Rankin, 1993; Murakami et al., 2005; Murakami and Murakami, 2005; Kauffman et al., 2010).

C. elegans show a variety of types of learning and memory (reviewed in Ardiel and Rankin, 2010); for example they show habituation of the tap withdrawal response (Rankin et al., 1990). Worms respond to non-localized mechanosensory stimuli (tap) by briefly changing from forward to backward locomotion (termed a reversal). The neural circuit that drives this behaviour is primarily composed of 5 mechanosensory cells, 8 interneurons and the motor neurons required for forward and backward locomotion (Wicks and Rankin, 1995). The distance they reverse in response to tap and the probability of responding with a reversal decreases with repeated stimulation (Rankin et al., 1990; Beck and Rankin, 1993). Preliminary work by Beck and Rankin (1993) investigated how tap habituation changed as worms aged and found that old worms (7 and 12 days of age; post-reproductive adults) showed greater habituation to tap stimuli presented at long interstimulus intervals (ISI) compared to younger worms. The goal of the current paper was to determine whether there are age related differences in habituation during the period of peak reproduction of the worm and, if there are, investigate their cause. In addition these studies provide a more thorough characterization of the effects of ageing on the behavioural parameters of habituation. We employ a newly developed automated behavioural tracking system, the Multi-Worm Tracker (MWT) (Swierczek et al., 2011), to monitor *C. elegans* behaviour. This system is capable of monitoring, scoring and analyzing the behaviour of 50-100 of worms at one time, making large parametric studies practical and rapid. This system also allows us to characterize

behaviour with more accuracy and detail than we previously could achieve with manual scoring techniques.

When reared at 20° C, *C. elegans* begin to lay eggs at ~ 70 hrs of age, with egg-laying peaking at 84 hrs of age. Egg-laying decreases dramatically between 96 to 108 hrs of age (Lindemans et al., 2009). We habituated worms at different points in the reproductive period, from the ages of 72 to 120 hrs post egg-lay, with taps delivered at both a 10 and 60s ISI with a range of stimulus intensities. The oldest worms that we assayed were at the end of their reproductive period but were 24 hrs younger than the age at which worms have been observed to show visible neurodegeneration of the touch receptor neurons (Pan et al., 2011).

My results extended the findings of Beck and Rankin (1993) by showing that as *C. elegans* age through their reproductive period they show an increasing capacity to show habituation of reversal probability in response to repeated stimuli of constant intensity. By manipulating stimulus intensity I was able to show that age-dependent changes in habituation were a reflection of worms decreasing abilities to discriminate between the intensity of sensory stimuli as they age. Finally, we optogenetically stimulated the touch receptor neurons; from this study our results suggest that the locus of these age-dependent changes are upstream of mechanosensory neuron depolarization.

4.2 Methods

4.2.1 Strains and maintenance

The reference, wild-type wild-type Bristol *C. elegans* strain was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN) and AQ2313 *Pmec-4*::ChR2::YFP; *Punc-11*::GFP was a gift from Dr. William Schafer (University of Cambridge, Cambridge, UK). Worms were cultured on Nematode Growth Medium (NGM)

seeded with *Escherichia coli* (OP50) as described previously (Brenner, 1974).

4.2.2 Behavioural testing

Worms were synchronized for behavioural testing by picking 5 gravid adults onto an OP50 *E. coli* seeded plate and letting them lay eggs for 3-4 hrs before they were removed. These eggs were allowed to hatch and worms to develop for 72-120 hrs (dependent upon the experiment) in a closed Tupperware box in a 20°C incubator. One hour prior to testing, 40-60 worms were transferred using a platinum pick to a fresh NGM plate. For experiments where worms were tested in the presence of food, plates were seeded with 20 µl of a liquid culture of OP50 *E. coli* 16-20 hrs beforehand. Plates of worms were placed into the tapping apparatus (Swierczek et al., 2011) and covered with an optically transparent lid constructed from a Petri plate lid, non-fogging cover-glass and wax. After a 100s acclimatization period, 30 taps were administered at either a 10 or a 60s inter-stimulus interval (ISI).

4.2.3 Image acquisition of behaviour

Stimulus delivery and image acquisition of the worms' behaviour was done using the MWT (version 1.2.0.2) (Swierczek et al., 2011). A Dalsa Falcon 4M30 camera (8 bits; 2352 x 1728 pixels, 31 Hz) and a Rodenstock 60 mm f-number 4.0 Rodagon lens was used to visualize a 5-cm Petri plate secured in the tapping apparatus. Images from the camera were captured using a National Instruments PCIe-1427 CameraLink capture card run along with the MWT tracking software on PC computers with Intel Core i3 2.93 GHz processors and 4 GB of RAM. The image of the plate was focused into the camera with a resolution of 0.027 mm/pixel. An elliptical region of interest was created ~5 mm from the inner edge of the Petri plate. Minimum and maximum object size thresholds were 80 and 400 pixels, respectively. The position, skeleton and outline of worms were acquired at a rate of 25 frames per second.

4.2.4 Channelrhodopsin-2 experiments

Channelrhodopsin-2 (ChR2) is a genetically encoded, blue light gated cation channel that can be used to precisely and noninvasively control the activity of well-defined neuronal populations (Boyden et al., 2005). ChR2 requires the co-factor all-trans-retinal (ATR), which was added to the NGM plates on which the worms were reared and tested. To make these plates we diluted ATR from a 100mM stock solution (in ethanol) to a 3mM working solution in liquid LB culture of OP50 *E. coli*. This culture was then spread onto NGM plates for a final concentration of 5µM ATR. These plates were stored in the dark at room temperature for 16-20 hrs before use in the rearing or testing of worms. For activation of ChR2, a custom built LED ring was used to illuminate plates with a uniform pattern of blue light (460-490 nm) at an intensity of 0.2mW/mm^2. Each stimulus was 250 ms in duration.

4.2.5 Behavioural scoring and statistical analysis

Offline data analysis was performed on a computer with a Intel Core i7-930 2.80 GHz processor with 6 GB of RAM using Choreography analysis software (version 1.3.0_r1035 software package) (Swierczek et al., 2011) to detect and measure the distance traveled during tap-evoked reversals. Choreography options "--shadowless", "--minimum-time 20", and "--minimum-move-body 2" were applied as filters. "--segment", "--plugin Reoutline", and "--Respine" were used to enhance detection of worm direction. "—plugin MeasureReversal::tap::dt=1::collect=0.5" was used to detect reversals within 1s of taps.

Two measures were analyzed: reversal probability (the number of worms that responded to tap with a reversal from all plates within the same experimental conditions summed and divided by the sum of the number of worms reversing and the number that did not respond) and reversal magnitude (reversal distance measured for worms that responded within 1 second to

tap). For both measures worms that were already reversing at the onset of the stimulus were omitted.

Reversal distances in response to either the initial or final tap of worms of various ages were compared by statistical analysis of covariance (ANCOVA), whereas response probability data was compared using a generalized linear mixed-model (GLMM) regression. Depending on the experiment, either age or stimulus intensity was modeled as a fixed effect. Petri plate (on which the worms were tested; minimum of 3 Petri plates of ~ 50 worms were tested per experimental condition) was modeled as a random effect nested within the fixed effect. To compare the two GLMM regression lines for 72 and 120 hr-old worms with stimulus intensity as the independent variable and probability of responding as the dependent variable ANCOVA was used. Planned comparisons were done using Tukey honestly significant difference (HSD) tests. For all statistical tests an alpha value of 0.05 was used to determine significance. Statistical analysis for initial response probability and reversal distance was only performed for 10s ISI experiments because the initial response probability and reversal distance data for 60s ISI experiments was considered to be equivalent and redundant. Matlab (version R2010b, Mathworks, Natick, MA, USA) was used for plotting graphs. ANCOVAs, Tukey's HSD post-hoc tests and GLMM regressions were performed using the statistical packages lme, lmer and glmmPQL in R (for Mac OS X GUI 1.40-devel Leopard build 32-bit).

4.2.6 Stimulus intensity

The mechanical tapper is composed of an electromagnetic tubular push solenoid (#195205-127, Ledex, Vandalia, OH, USA) that accelerates a plunger forward into the Petri plate and is pulled back into place by a spring (or elastic band for very weak stimuli). This was controlled by the MWT software through a PCI-bus compatible counter/timer board (#PCI-CTR05, Measurement Computing, Norton, MA, USA) with a solid state relay (#17M6585,

Newark, Chicago, IL, USA) powered by either a 15 or 25 V (dependent upon the experiment), 3 A, linear regulated AC-DC power supply (#A15MT300, Acopian, Easton, PA, USA). The kinetic energy (Ke) with which the plunger hits the plate with is dependent upon the force of the accelerating plunger multiplied by the distance the plunger travels from the starting position to the plate (Ke = force x distance). The force with which the plunger accelerates forward is dependent upon its starting position within the electromagnetic tubular push solenoid and the force exerted backwards by the spring or elastic that pulls the plunger back into the starting position. To measure the Ke with which the plunger hits the plate, I first measured the distance the plunger was from the Petri dish in each stimulus configuration and then multiplied this value by the sum of the forces acting on the plunger in the same stimulus configuration. To measure the forces acting on the plunger I used precision springs (#9433K38 and #9433K68, McMaster-Carr, Robbinsville, NJ, USA) to measure the force required (the distance we had to stretch the spring) to stop the plunger from accelerating forward from the starting position and subtracted from this the force the plunger is being pulled backward by the spring that returns the plunger to the starting position (the distance the spring is stretched when the plunger contacts the plate).

Laser Doppler vibrometry (LDV; OFV-5000, Polytec, Irvine, CA, USA) was used to study the vertical surface motion of the agar-filled Petri dishes caused by vibrations after a tap impacted the Petri dish. LDV is a non-contact method of recording vibrations that measures the velocity (m/s) or displacement (m) of a moving surface by detecting the Doppler shift of a reflected laser beam (Buchhave, 1975). This system was coupled into a Motic PSM-1000 microscope (Signatone Corp., Gilroy, CA, USA) and equipped with a displacement decoder (DD-200, Polytec, Irvine, CA, USA; best resolution: 2 nm, full scale output: +/- 82 mm, frequency range: 0 Hz - 250 kHz). The laser was focused on the surface of the centre of the Petri dish for all recordings. All recordings were made on a vibration-isolated table. Signals were recorded onto an oscilloscope (TPS200B, Tektronix, Beaverton, OR, USA) at a sampling rate of

1 GS/s. Maximum displacement (as a measure of stimulus intensity) was determined using Matlab.

4.3 Results

4.3.1 Short-term habituation in 72, 84, 96, 108 and 120 hour-old worms

I tested whether there was an effect of age on initial response to tap and final habituated level in groups of worms aged 72, 84, 96 108 and 120 hrs post egg-lay. To do this the behavioural metrics of reversal probability and reversal distance in response to 30 tap stimuli delivered at a 10 or 60s ISI were analyzed separately.

Reversal Probability: Age was not significantly related to the probability of responding to the initial tap (figure 4.1A, t=-0.78, d.f.=38, pNS) but was significantly related to the probability of responding to the final tap when stimulated at a 10s ISI (habituated level; t=-4.22, d.f.=38, p=0.0004). Next, worms of the same range of ages were habituated at a 60s ISI and again it was found that age was significantly related to the probability of responding to the final tap (figure 4.1B, t= -2.57, d.f.=39, p=0.01). A GLMM fitted with the collected data to model the relationship between age and final habituated level predicted that at a 10s ISI for every hr increase in age a 2.1% decrease in the probability of responding to the final tap should be observed. A similar, but weaker relationship was predicted for a 60s ISI (1.6% decrease/hr). Thus it appears that as worms age they habituate more deeply.

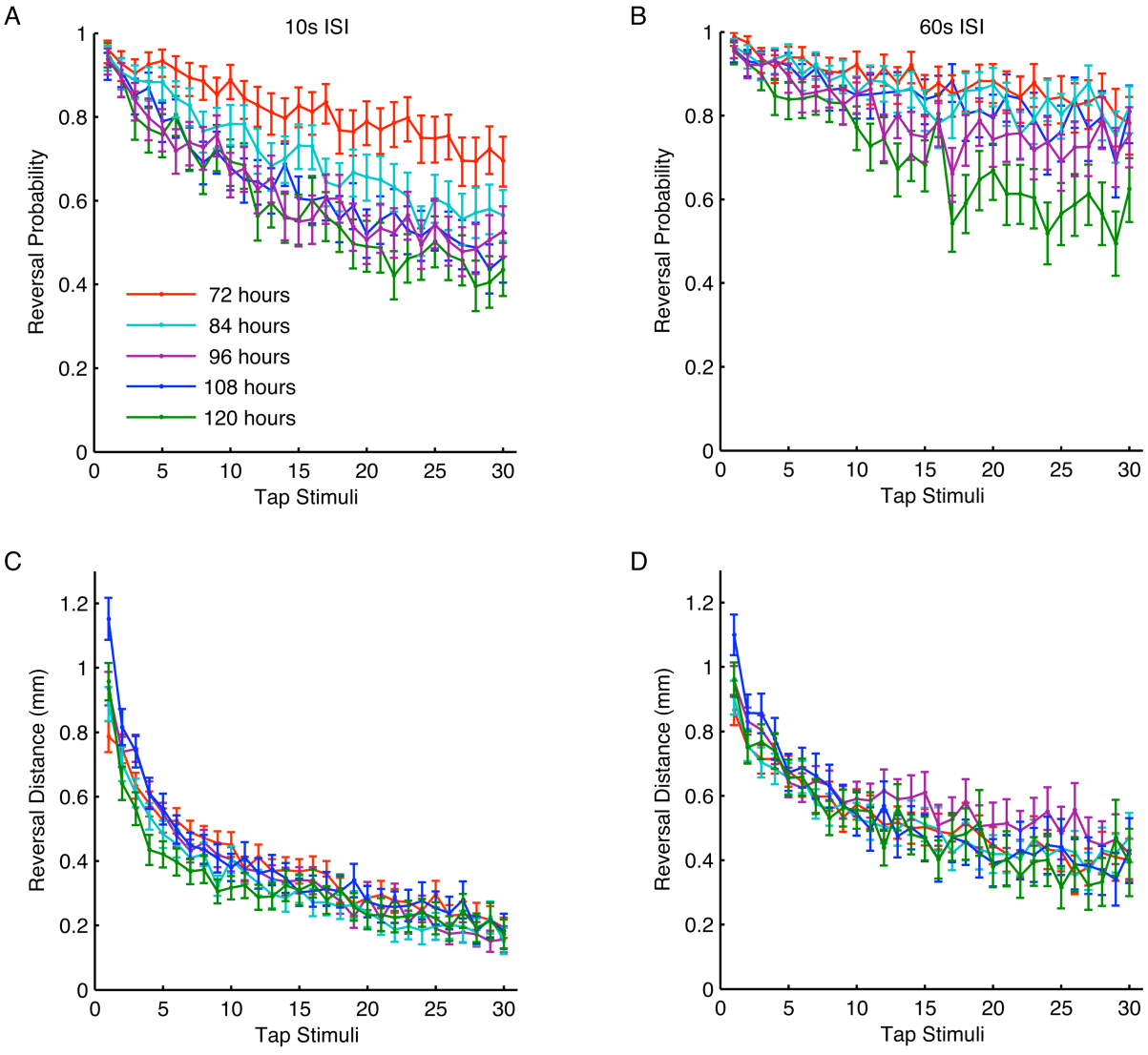

Figure 4.1 Tap habituation curves of 72, 84, 96, 108 and 120 hr-old wild-type *C. elegans* in response to a series of 30 taps tested in the presence of food.

Habituation curves for 72, 84, 96, 108 and 120 hr-old wild-type C. elegans in response to a series of 30 taps in the presence of food. There was an age-dependent effect of final habituated level for reversal probability at both a 10s ISI (a), and at a 60s ISI (b). There were no differences in final habituated level for mean reversal magnitude between different ages at either a 10s (c), or a 60s ISI (d). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

Reversal Distance: Unlike reversal probability, initial reversal distance to tap (figure 4.1C) was dependent upon age ($F(4,35)=7.57$, $p=0.0002$). When worms were habituated at a 10s ISI, 108-hr-old worms gave significantly larger reversals than did all other aged worms (108 hrs – 72 hrs, $p<0.001$; 108 hrs – 84 hrs, $p=0.01$; 108 hrs – 96 hrs, $p<0.001$; and 108 hrs – 120 hrs, $p=0.04$). Worm size and reversal speed increase linearly as worms age (data not shown) and thus could partially contribute to the increasing reversal distance in response to tap, but this does not sufficiently explain why 120 hr-old worms have significantly smaller responses to tap than 108 hr-old worms. There was no effect of age on final reversal distance level after habituation at either ISI (10s ISI, figure 4.1C, $F(4,35)=0.71$, pNS; 60s ISI, figure 4.1D $F(4,36)=0.40$, pNS).

4.3.2 Short-term habituation in 72, 96 and 120 hour old worms in the absence of food

I next tested whether the absence of food would alter the effects of age on habituation. As observed in other animals, behaviour in *C. elegans* can be modulated by its internal motivational state. Specifically, many behaviours (including tap habituation; Kindt et al., 2007) of *C. elegans* can be affected by their feeding state: whether or not the worms are assayed in the presence of food. To test whether this was also true for the age-dependent aspects of tap and tap habituation I performed tap habituation experiments in the absence of food at both a 10 and a 60s ISI on 72, 96 and 120 hr-old worms.

Reversal Probability: When food was absent, age was significantly related to the probability of responding to the initial tap (figure 4.2A, $t= -3.41$, $d.f.=21$, $p=0.003$). Similar to the first experiment (done on food), in the absence of food, age was again significantly related to the probability of responding to the final tap at both a 10s (figure 4.2A, $t= -5.38$, $d.f.=21$, $p<0.00001$) and 60s (figure4.2B, $t= -4.84$, $d.f.=39$, $p=0.0001$) ISI. For the probability of responding to the initial tap in the absence of food the GLMM predicts a 1.9% decrease for every

increase of hr in age. For final habituated level, at a 10s ISI the GLMM predicts a 3.1% decrease/hr in the probability of responding to the 30th tap. At a 60s ISI the GLMM predicts a 3.8% decrease/hr.

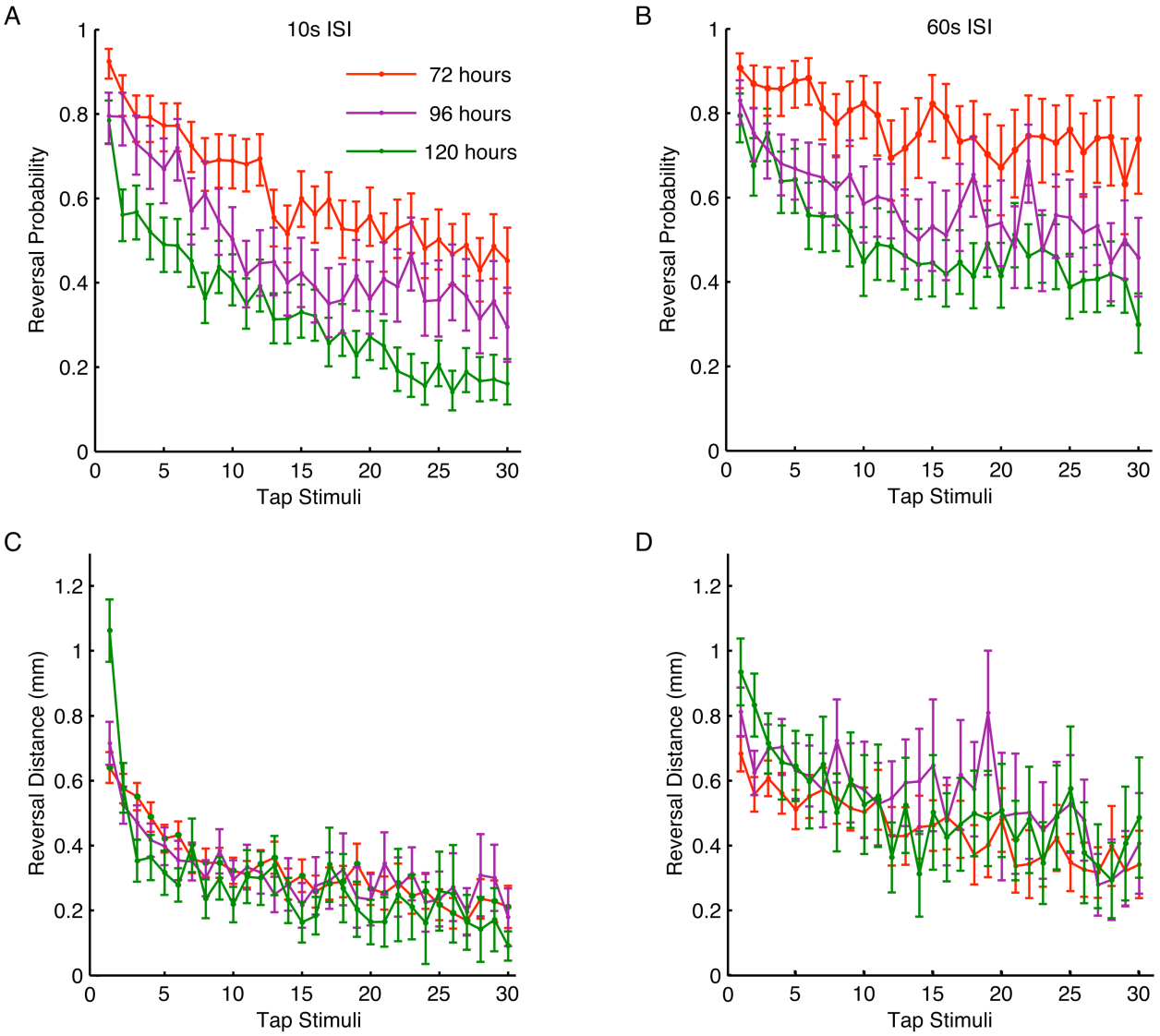

Figure 4.2 Tap habituation curves of 72, 96, and 120 hr-old wild-type *C. elegans* in response to a series of 30 taps tested in the absence of food.

Habituation curves for 72, 84, 96, 108 and 120 hr-old wild-type C. elegans in response to a series of 30 taps in the absence of food. There was an age-dependent effect of final habituated level for reversal probability at both a 10s ISI (a), and at a 60s ISI (b). There were no differences in final habituated level for mean reversal magnitude between different ages at either a 10s (c), or a 60s ISI (d). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

To test if the effect of age on response probability habituation was altered by the presence or absence of food we compared the slopes of two regression lines (one for the presence of food and one for the absence of food) when the probability of responding to the final tap was modeled as the dependent variable and age was modeled as the independent variable. When we did this for experiments where the worms were habituated at a 10s ISI we found that the slopes of the two regression lines were not significantly different (pNS), but the Y-intercepts of these two regression lines were significantly different ($p<0.0001$), meaning that for any given age of worm, in the presence of food worms are significantly more likely to respond to the final tap than are worms that are habituated in the absence of food. In contrast, for experiments where the worms were habituated at a 60s ISI when we compared the two regression lines we found that the slopes were significantly different ($p=0.03$); age had a larger impact on reversal probability of the last tap for the worms habituated in the absence of food compared to worms habituated in the presence of food. In summary, when worms are habituated at a 60s ISI the absence of food magnifies the effect of age on response probability habituation.

Reversal Distance: Similar to experiments performed in the presence of food, initial reversal distance to tap tested in the absence of food was dependent upon age (figure 4.2C, $F(2,20)=9.94$, $p=0.005$). The oldest worms, 120 hrs old, worms gave significantly larger reversals than did all other aged worms (120 hrs – 72 hrs, $p=0.001$; and 120 hrs – 96 hrs, $p=0.02$). Increased body size and reversal speed with age may explain this effect (data not shown). There was no effect of age on final reversal distance habituated level at either ISI (10s ISI, figure 4.2C, $F(2,20)=2.47$, pNS; 60s ISI, figure 4.2D, $F(2,20)=0.82$, pNS).

4.3.3 Habituation to optogenetic stimulation of the touch receptor neurons in 72 and 120 hr-old worms

In an attempt to localize the site of the age-related changes in habituation of response probability the touch receptor neurons were directly depolarized. The genetically encoded blue light gated cation channel Channelrhodopsin-2 (ChR2) was transgenically expressed in the touch cells in one strain of worms. If fed the essential opsin co-factor ATR, the majority of these worms swam backwards in response to a 250 ms blue light pulse. Wild-type worms or ChR2 transgenic reared in the absence of ATR did not respond to the blue light (data not shown); indicating that the blue light induced reversals were caused by activation of ChR2 and not an innate blue light avoidance response. In addition, the presence of ATR did not alter wild-type responses to tap (data not shown). I predicted that if the age-dependent changes in behaviour are caused by mechanisms downstream of depolarization of the mechanosensory neurons (e.g. differences in neurotransmitter release from the sensory neurons or the interneurons) then activating them with blue light should show similar effects to tap. If however the age-dependent changes in behaviour are caused by mechanisms upstream of depolarization (e.g. differences in mechanotransduction) then they should not be apparent in responses elicited by ChR2 activation. We habituated plates of 72 and 120 hr-old *Pmec-4*::ChR2::YFP worms with 30 blue light stimulations at a 10s ISI.

Reversal Probability: As with mechanical tap, age was not significantly related to the likelihood of responding to the initial light pulse (figure 4.3A, t= -1.29, d.f.=14, pNS). In contrast to tap, however, age was not significantly related to the probability of responding to the final light pulse (figure 4.3A, t= 2.03, d.f.=14, pNS).

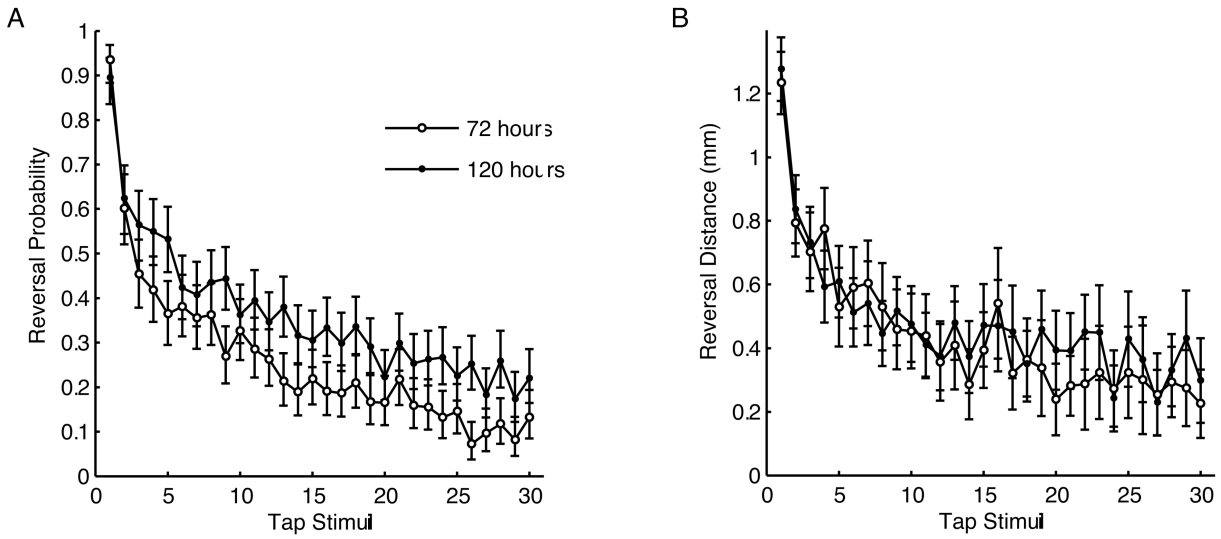

Figure 4.3 Blue-light habituation curves of 72 and 120 hr-old *C. elegans* (tested in the presence of food) expressing ChR2 in the touch receptor neurons in response to a series of 30 lights given at a 10s ISI.

Blue-light habituation curves for 72 and 120 hr-old C. elegans (food present) expressing ChR2 in the touch receptor neurons in response to 30 lights at a 10s ISI. No age-dependent differences in final habituation level were observed for either reversal probability (a) or mean reversal magnitude (b). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

Reversal Distance: Neither initial nor final reversal distance was dependent on age when worms were stimulated via ChR2 at a 10s ISI (figure 4.3B; $F(1,14)=0.43$, pNS and $F(1,13)=0.51$, pNS, respectively).

4.3.4 Habituation to taps of differing intensities in 72 and 120 hour-old worms

The ChR2 experiments suggested that the site of age-dependent changes in behaviour was located upstream of depolarization of the touch receptor neurons and perhaps caused by changes in the transduction of the mechanosensory stimulus. One behavioural characteristic of habituation (observed from protozoans to mammals) is that within a stimulus modality, weaker

stimuli cause more rapid and/or deeper response decrements, while stronger stimuli cause slower and/or shallower response decrements (Harris, 1943; Kuenzer, 1958; Prechtl, 1958; Uno and Grings, 1965; Thompson and Spencer, 1966; Groves and Thompson, 1970; Fischbach and Bausenwein, 1988; Rankin et al., 2009) (although not always the case (Dunlop et al., 1964; Miller and Murray, 1966; Wolfensberger and O'Connor, 1967; Davis and Wagner, 1968; Askew, 1970)). Therefore I hypothesized that one reason for both previous (Beck and Rankin, 1993) (1993) and current findings might be that older adults may be less sensitive to mechanical stimuli than younger adults, i.e. older adults habituate to a deeper level because they perceive the mechanical stimuli as a less intense stimulus than do younger worms. To investigate this hypothesis Laser Doppler vibrometry (LDV) was first used to quantify the amplitude (measured as maximal displacement of the vibrational wave created in the agar by the tap stimulus) of several different tap stimuli administered using our apparatus. Because the LDV system I had access to was mounted on and focused through a microscope we could only accurately measure tap-induced vibrations of < 82μm. Because our standard tap stimulus (the one used in experiments described above) created a maximum displacement that was > 82μm, I plotted the kinetic energy of impact against the max. displacement for those stimulus strengths that were measured. From this standard curve I was able to estimate the max. displacement created by the standard tap used in our lab to be 113.8μm (in this setup the tapper hits the plate with 18.0 mJ of kinetic energy; figure 4.4). I then tested the initial response and final habituation level (at a 10s

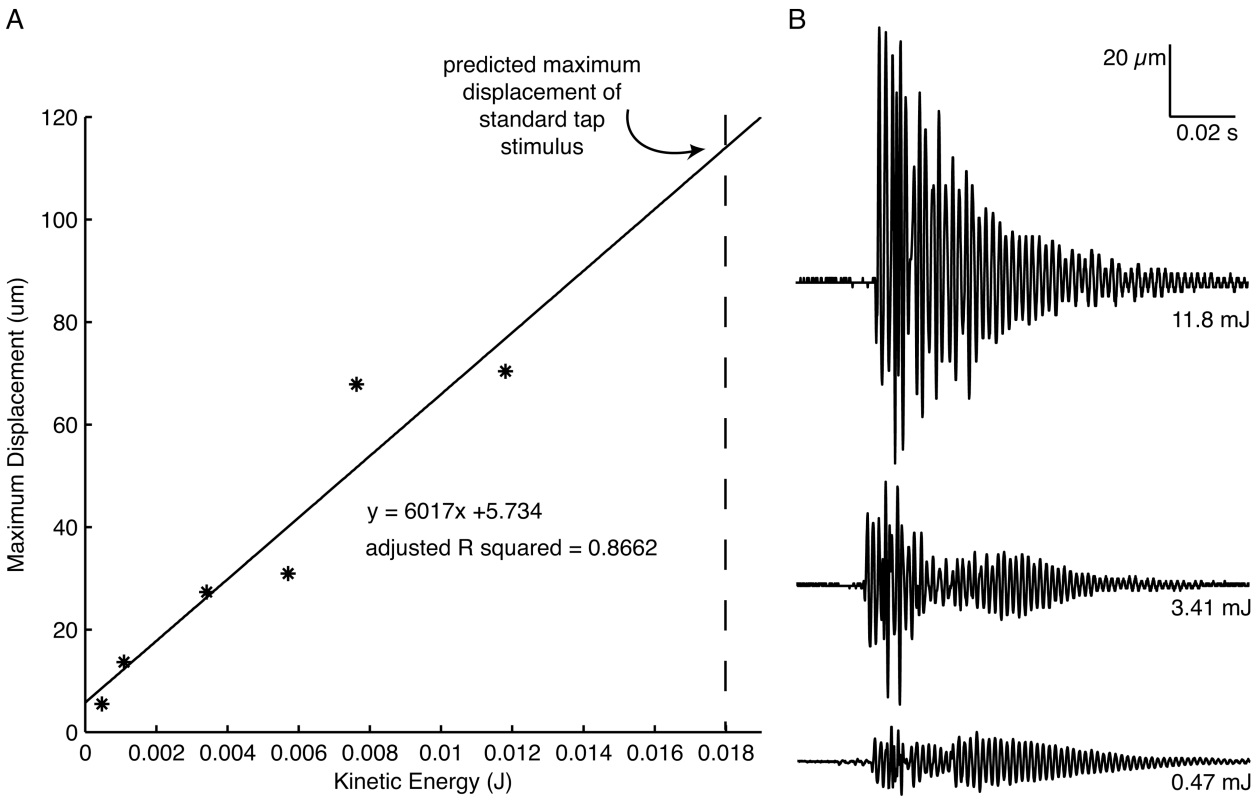

Figure 4.4 Quantification of intensity of tap stimulus.

A) Correlation between maximum displacement of the vertical component of the vibration of the agar surface created by the tap stimulus as measured by laser Doppler vibrometry (LDV) and the kinetic energy the tapping apparatus hit the side of the Petri dish with. Each data point represents the mean maximum displacement from 3 LDV recordings. B) Representative LDV recordings of vertical displacement of agar surface of Petri dish. Top trace was tap of greatest intensity that could be recorded and the bottom trace was the weakest intensity tap we could reliably create. Inset beneath each trace is the measured kinetic energy that the tapping apparatus hit the Petri plate with.

ISI) of 72 and 120 hr old worms in response to four calibrated intensities (taps that created a max. displacement of 5.5, 19.4, 30.9 and 113.8μm).

Reversal Probability: Age was a significant factor in predicating the probability of responding to the initial taps of different stimulus intensities. The slopes of the two regression lines (one for 72 hr-old worms and one for 120 hr-old worms) when probability of responding to the initial tap was modeled as the dependent variable and stimulus intensity was modeled as the

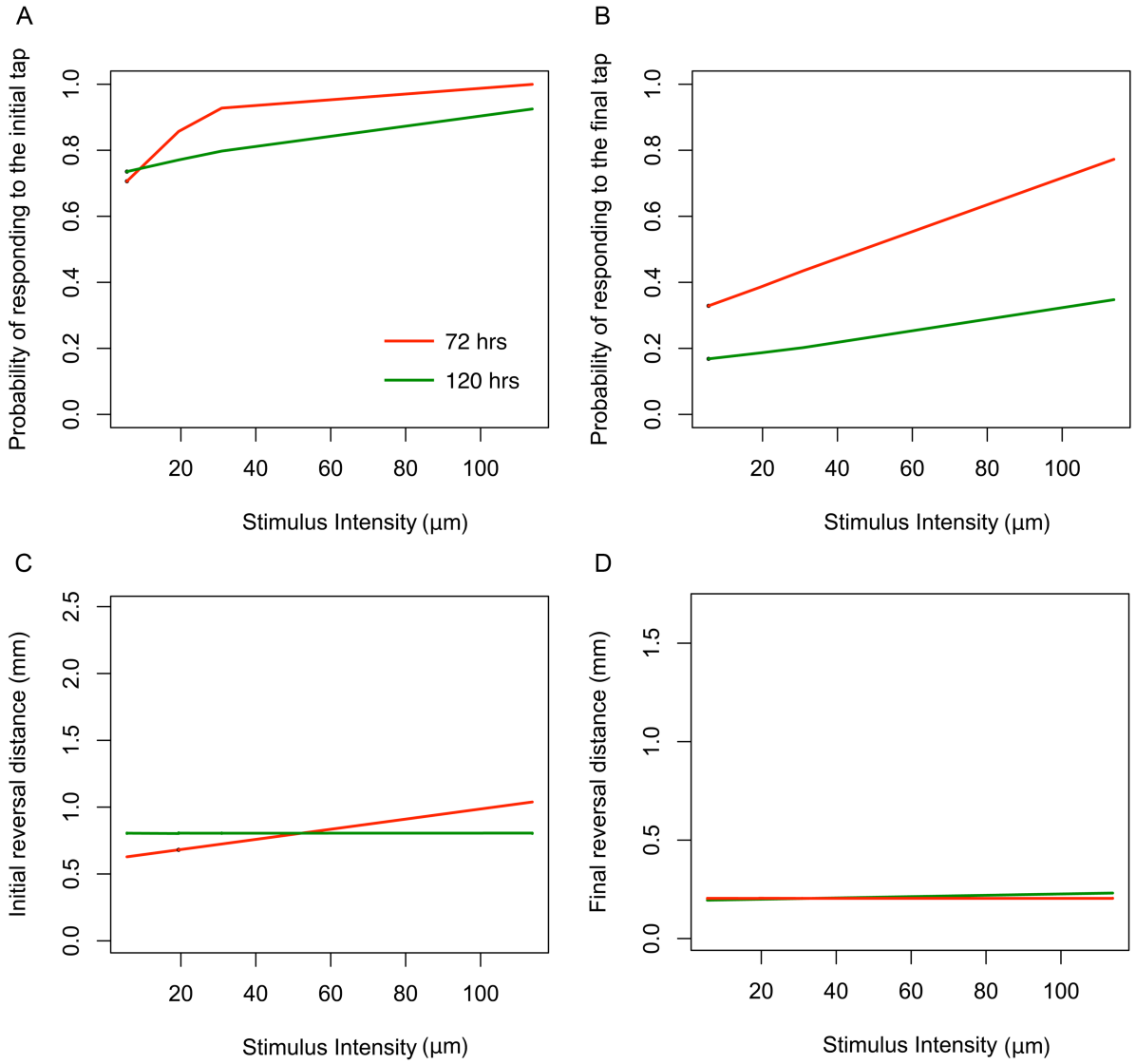

Figure 4.5 Regression analysis of stimulus intensity and tap and tap habituation of 72 and 120 hr-old *C. elegans*.

A) 72 hr-old worms' probability of responding to the initial tap increases at a greater rate as stimulus intensity compared to 120 hr-old worms. B) 72 hr-old worms' probability of responding to the final tap (habituated level) increases at a greater rate as stimulus intensity compared to 120 hr-old worms. C) 120 hr-old worms give larger responses to tap than do 72 hr-old worms (except for the 113.8 µm stimulus). D) No difference in reversal distance in response to the final tap was observed between 72 and 120 hr-old worms.

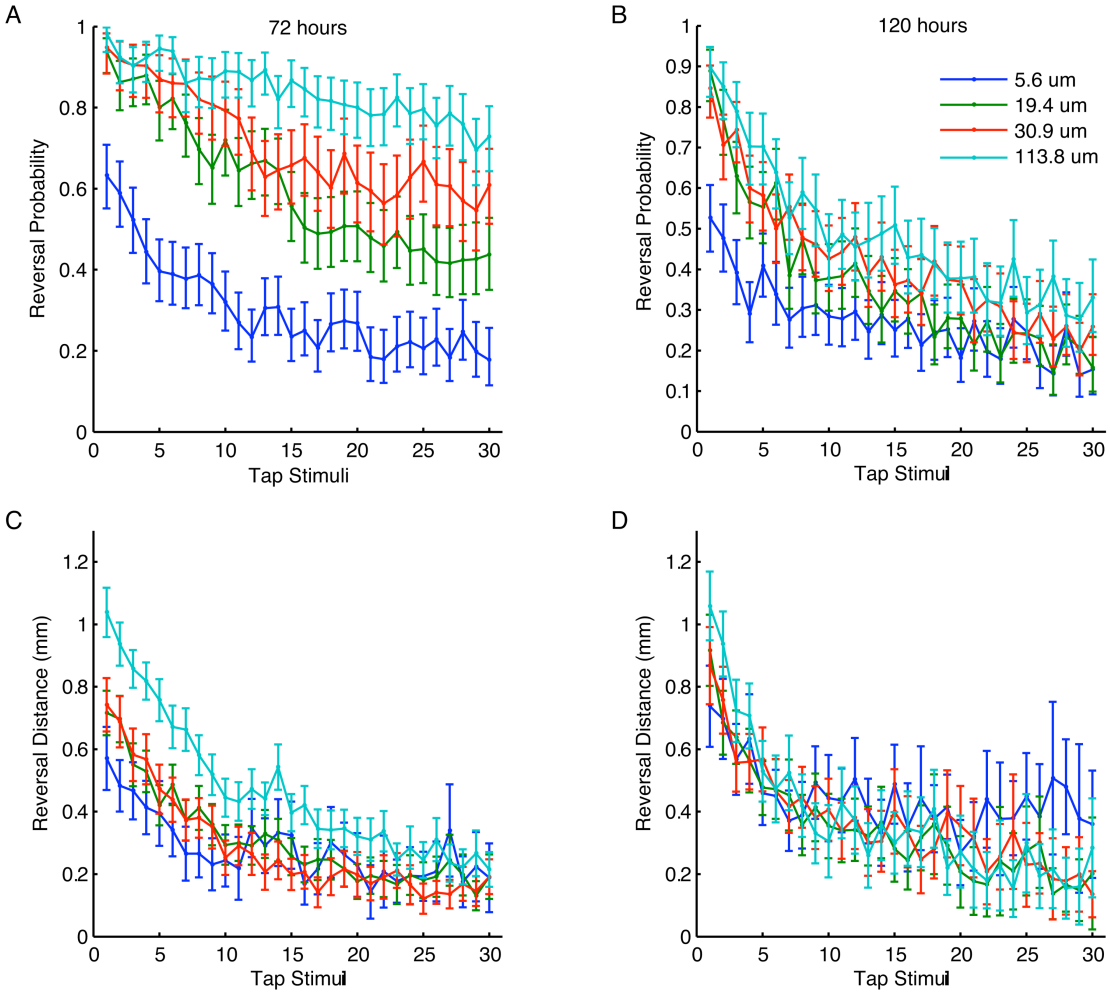

Figure 4.6 Tap habituation curves of 72 and 120 hr-old wild-type *C. elegans* (tested on food) in response to a series of 30 taps of 4 different intensities (measured as maximum displacement).

Habituation curves for 72 and 120 hr-old C. elegans (food present) in response to 30 taps at a 10s ISI for 4 different tap intensities (measured as maximum displacement). Final habituated level for reversal probability was dependent on tap intensity. This relationship was stronger in 72 hr-old worms (a) than in 120 hr old worms (b). For mean reversal magnitude there were no differences in final habituated level to different intensity taps at either age (c) & (d). Error bars represent 95% confidence intervals (Clopper-Pearson method for binomial data).

independent variable were significantly different (p=0.005): 72 hr-old worms were more sensitive to changes in stimulus intensity than 120 hr-old worms (figure 4.5A). Both 72 and 120 hr-old worms had a lower probability of responding to the weakest stimulus that we could deliver (5.5µm max. displacement, p<0.0001 for both ages, figure 4.6A and 4.6B) compared to all other intensities. All other comparisons were not significant (pNS).

Age was a significant factor in predicating the probability of responding to the final taps of different stimulus intensities. The slopes of the regression lines for 72 and 120 hr-old worms were significantly different ($p=0.03$); stimulus intensity had a larger impact on reversal probability of the last tap for the 72 hr-old group compared to the 120 hr-old group (figure 4.5B). Within the 72 hr age group, the weakest stimulus, 5µm max. displacement, resulted in significantly greater habituation than did any other stimulus (figure 4.6A, $p<0.0001$). The next strongest stimulus, 19.4µm max. displacement, also resulted in significantly greater habitation than the next two stimuli of greater intensity (30.9µm max. displacement, $p=0.04$ and 113.8µm, $p<0.0001$). The difference in probability of responding to the final tap between the 30.9µm and 113.8µm stimulus seen in figure 4a was not significant (pNS). In contrast, within the 120 hr age group only the weakest stimulus, 5µm max. displacement, resulted in a significantly lower probability of responding to the final tap compared the strongest two stimuli (30.9µm and 113.8µm max. displacement, figure 4.6B, $p<0.01$), all other comparisons were not significant (pNS). In summary, although both 72 hr-old and 120 hr-old worms modulate initial and final reversal probability in response to varying stimuli, the younger animals do so to a significantly greater extent.

Reversal Distance: Age was not a significant factor in predicating the reversal distance in response to the initial taps of different stimulus intensities. The slopes of the two regression lines (one for 72 hr-old worms and one for 120 hr-old worms) when reversal distance in response to the initial tap was modeled as the dependent variable and stimulus intensity was modeled as the independent variable were not significantly (pNS). The Y-intercepts of these two regression lines were significantly different ($p=0.0004$) meaning that 120 hr-old worms respond with significantly greater reversal distances to a stimulus of any given intensity than do 72 hr-old worms (figure 4.5C).

Post hoc comparisons within 72 hr-old worms revealed that they responded with significantly larger reversals to the strongest stimulus (113.8µm max. displacement) compared to all other stimulus intensities (figure 4.6C, $p<0.0001$), whereas post hoc comparisons within 120 hr-old worms revealed that a significant difference was only observed between the strongest and the weakest stimulus intensities (figure 4.6D, $p=0.004$). Thus it appears that with respect to reversal distance elicited by the initial response to tap, 72 hr-old worms were slightly more sensitive to changes in stimulus intensity than were 120 hr-old worms.

In contrast, no age-dependent differences were observed once the worms were habituated at different stimulus intensities. Neither the slopes of the two regression lines nor the Y-intercepts were significantly different (pNS). Thus worms of both ages habituated to the same level of reversal distance regardless of the stimulus intensity.

4.4 Discussion

In summary these findings support the hypothesis that *C. elegans* exhibit age-dependent changes in habituation and that these changes are not limited to post reproductive age worms (≥ 7 days old; Beck and Rankin, 1993) but are also apparent as worms age during the reproductive period. Specifically, as worms age from young adult (72 hrs of age) to late adult (124hrs) they are less likely to respond to the final tap in a habituation series (i.e. older worms habituate to a deeper level). This effect is enhanced when worms are tested in the absence of food. Interestingly, no age-dependent change in the habituation of reversal distance was seen. In contrast to tap habituation, when blue-light instead of taps was used to stimulate the mechanosensory neurons of young adult and older worms expressing ChR2 in the touch cells an age-dependent difference in habituation of reversal probability was not observed, suggesting age-related changes occur upstream of depolarization of these cells. Finally, the negative relationship

between age and final habituated level in response probability was found to be associated with the worms decreasing response to differences in stimulus intensities as they age.

Assaying habituation in *C. elegans* using the MWT has allowed a large parametric behavioural study presenting many stimuli at five different intensities to hundreds of animals at a range of ages to be performed. I observed a strong relationship between stimulus intensity and the probability of responding to the final tap in a habituation series in 72 hr-old worms. I found that changing stimulus intensity caused a large and significant change in the final habituated level. This finding is consistent with the literature: less intense stimuli cause more habituation while more intense stimuli cause less habituation (Harris, 1943; Kuenzer, 1958; Prechtl, 1958; Uno and Grings, 1965; Thompson and Spencer, 1966; Groves and Thompson, 1970; Fischbach and Bausenwein, 1988; Rankin et al., 2009). In older worms (120 hr-old) a relationship between stimulus intensity and the likelihood of responding to the final tap in a habituation series was almost absent. Only a stimulus of just above threshold intensity (< 60% of animals responded to the initial presentation of the stimulus) was able to significantly alter the likelihood of responding the final tap in a habituation series for 120-hr-old worms. Thus, these findings of age-dependent changes in final habituated level of response probability may be due to the failure of older worms to discriminate behaviourally between different intensities of mechanosensory stimuli (although they can discriminate between stimuli of grossly different intensities).

Age-dependent changes in learning and memory have been observed in a wide variety of animals but in most instances there is no clear understanding of the mechanism by which it happens. One possibility suggested by these experiments is that age-related cognitive deficits might be caused by modest changes in stimulus discrimination that begin during middle age. One can imagine that as stimulus discriminatory abilities decline (and as a consequence, stimulus generalization increases) learning about a variety of stimuli in our environment could be compromised. In fact, many psychophysical studies have shown a loss in stimulus frequency

discriminatory abilities (Konig, 1957; Abel et al., 1990; Moore and Peters, 1992; He et al., 1998; Espinoza-Varas and Jang, 2006; He et al., 2007; Clinard et al., 2010) and stimulus intensity discriminatory abilities with age (Humes and Christopherson, 1991; Christopherson and Humes, 1992; Humes, 1996; He et al., 1998). The conservation of age-dependency of deficits in sensory discriminatory abilities from *C. elegans* to humans suggests that the cellular mechanisms for this may also be conserved. *C. elegans* is a model organism with a tractable nervous system that is amendable to high-throughput behavioural and genetic experimentation. Developing it as a model to study age-related decline in stimulus intensity discrimination for sensations may provide insight into how these changes occur.

Our optogenetic experiments suggest that the cellular mechanism for these age-dependent changes in behaviour likely lies upstream of depolarization of the touch receptor neurons. Potential changes could include thickening of the cuticle, changes in the strength of attachment of the mechanosensory cells to the body wall, changes in the length/width ratio of the mechanosensory cells themselves (which could cause changes in input resistance) and/or changes in expression of genes responsible for mechanotransduction or signal amplification. O'Hagan and colleagues (2005) performed *in vivo* whole-cell patch clamp recordings of the mechanosensory cells in *C. elegans* in their third and fourth larval stages (L3 and L4 respectively). In these very young worms increasing the intensity of the mechanical stimulus resulted in an increase of the amplitude of the mechanoreceptor current. This experiment has not been done for *C. elegans* of older ages but it would be interesting to know if the mechanoreceptor current amplitude became less sensitive to changes in the intensity of the mechanical stimulus as this could potentially explain at least part of the age-related behavioural changes I observed.

Although the majority of ageing studies have been performed in late adulthood in human studies, there have been some studies that have looked at earlier time points. For example: 1)

Larrabee and Crook (1994) found that age-associated memory impairments measured via standard clinical and computer simulated everyday memory tests begin between 30 and 40 years of age, 2) Park and colleagues (1996) found that long-term declarative memory and working memory begin to decline between 20 and 30 years of age and 3) Salthouse (2009) investigated the effects of ageing on reasoning, spatial visualization, episodic memory and processing speed and observed that some aspects of age-related cognitive decline started as early as 20 and 30 years of age in healthy adults. In addition, a number of neurobiological variables have been observed to exhibit age-associated declines beginning in adults as young as 20 years old (brain volume & cortical thickness (Magnotta et al., 1999; Sowell et al., 2003; Allen et al., 2005; Fotenos et al., 2005; Salat et al., 2005; Kruggel, 2006; Pieperhoff et al., 2008), myelin integrity (Sullivan and Pfefferbaum, 2006; Hsu et al., 2008), neuromodulator receptor binding (Volkow et al., 2000; Sheline et al., 2002; Erixon-Lindroth et al., 2005), accumulation of neurofibrillary tangles (Del Tredici and Braak, 2008), and brain metabolites concentrations (Kadota et al., 2001)). Both humans and worms exhibit evidence of age-related changes in behaviour that begins during early adulthood and because any underlying genetic mechanisms may be conserved, our findings suggest mechanosensory habituation in *C. elegans* is a good model for age-related decline that begins in early adulthood.

This study extends the findings of Beck and Rankin (Beck and Rankin, 1993) by demonstrating that age-dependent changes in short-term habituation 1) start as early as 84 hrs (when raised at 20° C) and occur during the worms reproductive period 2) are due to changes in response probability and not reversal distance at both short and long ISIs, 3) occur in the presence of food and are enhanced when worms are tested in the absence of food and 4) are at least in part mediated by decreases in the ability of *C. elegans* to discriminate mechanosensory stimuli of different intensities as they age. These data also suggest the hypothesis that the site of these changes is upstream of depolarization of the mechanosensory neurons. Further research

should be directed at identifying the anatomical and/or physiological changes responsible for the age-dependent changes in mechanosensitivity and response habituation.

CHAPTER 5: General Discussion

5.1 Long-term memory, including that of habituation, is CREB-dependent

In Chapter two, I report on the role of CREB in long-term habituation. Specifically I found that the single *C. elegans* homologue of CREB, *crh-1*, was necessary for long-term habituation to tap. This phenotype was specific to long-term memory: *crh-1* was not necessary for learning or other forms of memory. Expressing a wild-type copy of CRH-1 in *crh-1* mutants in the reversal interneurons of the tap withdrawal circuit (AVA and AVD) and not the mechanosensory neurons rescued the long-term habituation defects, suggesting that long-term memory for tap habituation in *C. elegans* is at most localized in these two pairs of neurons.

A paper published several months after mine replicated our findings in *Drosophila*. Das et al., (2001) found that brief induction of an inhibitory form of CREB (CREB2b) in multiglomerular local interneurons blocked long-term habituation, but had no effect on short-term habituation. These data combined with ours suggest that CREB's function in interneurons of behavioural circuits for long-term habituation is conserved across phylogeny, at least in invertebrates.

The first evidence that hinted that CREB might play a role in mediating the transcription necessary for induction of long-term plasticity came from studies in a primary neuron coculture system used to study the facilitation of synaptic transmission between sensory and motorneurons comprising the monosynaptic component of the gill withdrawal reflex in the sea hare, *Aplysia californica.* Using this reduced preparation to study long-term synaptic facitilation, Dash and colleagues (1990) injected sensory neurons with oligonucleotides comprised of CRE's and found that when they tried to induce long-term facilitation, this manipulation blocked its induction. Further studies in *Aplysia* confirmed that CREB was in fact the protein responsible for long-term facilitation. They were able to induce expression of a lacZ reporter gene driven by the CRE by applying five 5 min pulses of 5-HT over 1.5 hrs (a protocol known to induce long-term

facilitation) and used GAL4-CREB fusion genes to show that 5-HT induction of CRE-lacZ expression requires phosphorylation of CREB by protein kinase A (Kaang et al, 1993).

Not long after this initial finding, the importance and conservation of CREB's role in long-term memory was made clear. Yin et al (1994) tested whether transgenic fruit flies, *Drosophila melanogaster*, expressing a dominant negative form of CREB-2b under control of a heat shock promoter could form long-term memory of Pavlovian olfactory learning. Flies that were administered heat shock to induce the dominant negative form of CREB before long-term memory training were unable to show memory of this training in a memory test 24 hours later, whereas flies that were left at the restrictive temperature before training were able to. CREB's requirement for long-term memory in mice was demonstrated in the same issue of the journal Cell: Bourtchuladze et al. (1994) showed that long-term potentiation and long-term memory, but not short-term memory, for both fear conditioning and the Morris water maze was absent in mice with a targeted disruption in the α and δ isoforms of CREB. These were the first demonstrations that CREB was required for long-term memory *in vivo*. These findings have been extended to long-term memory for avoidance learning in rats (Bernabeu et al., 1997), long-lasting synaptic plasticity in pond snails (Sadamoto et al., 2004), long-term memory for associative learning in *C. elegans* (Kauffman et al., 2010), and I used *C. elegans* to demonstrate for the first time the requirement of CREB for long-term habituation in chapter two.

Although it appears CREB plays a critical role in long-term memory of many forms, from habituation to sensitization to associative learning, in all species, from simple invertebrates to mammals, unresolved questions about CREB's role in long-term memory are: what genes does CREB transcribe and what function do they have in modifying the cell and/or synapses to induce memory? There is evidence that suggests that a target of CREB during long-term memory induction might be its own gene. Many biochemical investigations of temporal CREB activity (assayed by its phosphorylation of Ser 133) after training in different types of learning tasks or

stimulations known to induce long-term synaptic changes have observed a biphasic (Bernabeu et al., 1997; Schulz et al., 1999; Stanciu et al., 2001; Ahmed and Frey, 2005) or prolonged period (Taubenfeld et al., 1999; Leutgeb et al., 2005; Liu et al., 2008) of CREB activation and in *Aplysia*, CREB1 was observed to bind to its own promoter (Liu et al., 2008). These studies suggest the possibility that there may be a positive-feedback loop where phosphorylated CREB might induce its own gene via CREs to prolong its activity.

C/EBP, an immediate-early gene (Alberini et al., 1994; Niehof et al., 1997; Sterneck et al., 1998; Lee et al., 2001; Taubenfeld et al., 2001; Chen et al., 2003), and brain derived neurotrophin factor (BDNF, Barco et al., 2005) are examples of other genes known to be required for long-term synaptic facilitation and long-term memory that are likely to be transcribed by CREB. CREB can be activated by hundreds of different stimuli, aside from those that induce long-term memory, and because there are hundreds of CRE sequences in the genome it likely that there are more targets of CREB. Recent advances in genomics could be useful to try to identify the genes activated by CREB in a long-term habituation paradigm. My finding that CREB is required in AVA and AVD for long-term habituation in concert with data mining from the National Human Genome Research Institute model organism ENCyclopedia Of DNA Elements (modENCODE) project could serve as a useful starting point to determine additional novel CREB targets. The goal of the modENCODE project is to identify all of the sequence-based functional elements in the *Caenorhabditis elegans* and *Drosophila melanogaster* genomes including cis-elements to which transcription factors bind. This data could be used to generate a list of evolutionarily conserved genes that have CRE elements in their promoter regions. This list could then be cross-referenced with the known gene expression of AVA and AVD from the literature (accessible on wormbase.org) and the gene expression of AVA from the results of a recent study by Spencer and colleagues (2011) who performed tiling arrays to develop gene expression profiles of specific cells by using tissue specific promoters to mark cells for mRNA

extraction by the mRNA tagging method. The resulting reduced list of candidates expressed in AVA and AVD could then be screened for long-term habituation deficits in *C. elegans*. Genetic rescue experiments could be performed to confirm that any genes whose mutation results in the inability to form long-term memory is a target of CREB. If expressing the gene under control of a wild-type copy of its endogenous promoter in the mutant strain rescues the long-term memory defect then it should be tested if expressing the gene under control of a mutated copy of its endogenous promoter where the CRE has been altered to inhibit CREB binding will also rescue long-term habituation. If it cannot, then that would demonstrate that CREB promotes transcription of that gene during the induction long-term habituation. This approach would allow the identification of novel CREB targets and would greatly enhance our understanding of long-term memory.

There are two major limitations of this approach. The first is that the expression profiles of many genes in *C. elegans* are still not known and thus using the expression data available from wormbase.org will leave out many potential candidates. Secondly, trying to address the first limitation by including data from the tiling arrays of Spencer and colleagues (2011) will still lead to the omission of genes that are expressed at very low levels, are not expressed in young adult worms (the age of worms assayed by Spencer et al., 2001) and/or are only expressed after long-term memory training.

5.2 Short-term habituation and long-term habituation, a serial or parallel process?

One of the questions that the data in this work raises is about the relationship between short- and long-term memory. Traditionally they have been thought of as serial processes in which short-term memory is consolidated and becomes long-term memory. Generally, across a wide variety of species and learning paradigms, most manipulations that alter/impair short-term memory also cause impairment of long-term memories; however, there are a few exceptions

where there is evidence that shows that sometimes short-term and long-term memories are not serial processes but exist in parallel. For example: facilitation in *Aplysia* (Emptage and Carew, 1993), inhibitory avoidance training in rats (Izquierdo et al., 1998), and appetitive learning in *Drosophila* (Trannoy et al., 2011). In these studies researchers were able to dissociate short- and long-term memory; they showed that long-term memory could be formed without the expression of short-term memory.

In chapter two, I report that CMK-1 is required for wild-type long-term habituation but I also report in chapter three that CMK-1 is critical for short-term habituation at a 60s ISI. Because the protocol used to induce long-term habituation is comprised of spaced training within which stimuli are delivered at a 60s ISI (Rankin et al., 1990; Rose et al., 2002) it could be that *cmk-1* mutants do not show long-term habituation only because they do not habituate as deeply as wild-type worms at a 60s ISI.

Unlike vertebrate CaMK1, *C. elegans* CMK-1 includes a nuclear localization signal at the N-terminus of the protein that allows it to enter the nucleus of the cell (Eto et al., 1999) where it could act to phosphorylate transcription factors such as CREB. It is also expressed in the cytoplasm (Eto et al., 1999; Satterlee et al., 2004) where it could act to phosphorylate proteins that modulate cell excitability and synaptic release. Based on these observations it is possible that CMK-1 functions in the nucleus to phosphorylate CREB during long-term habituation induction and functions in the cytoplasm to modulating cell excitability and/or synaptic release during short-term habituation. To test this hypothesis the *cmk-1* mutants could be rescued with CMK-1 cDNA that does not contain the nuclear localization signal: creating a worm strain that expresses CMK-1 only in the cytoplasm. This strain could then be tested for short- and long-term habituation. I predict that if CMK-1 functions independently in both short- and long-term habituation this strain should show normal short-term habituation but should be unable to form

long-term habituation even though they should habituate as deeply as wild-type worms in the measure of reversal distance and reversal magnitude.

Short-term habituation is thought to occur in the mechanosensory cells (Wicks and Rankin, 1997) whereas in chapter two I have shown that long-term habituation occurs in the reversal command interneurons of the tap withdrawal circuit (AVA and AVD). Thus if CMK-1 plays a role in both short and long-term habituation it could also be that it's functions in these two separate processes are in two different cellular locations within the nervous system: CMK-1 may function in short-term habituation in the mechanosensory neurons and function in AVA and/or AVD in long-term habituation. Rescuing or knocking down CMK-1 in different portions of the tap withdrawal circuit would help determine whether the short- and long-term habituation deficits have the same or different loci.

If short-term and long-term memory for tap habituation in *C. elegans* is a serial process and CMK-1 mutants do not form long-term memory of habituation training because they have impaired short-term habituation at a 60s ISI, then the next question is which aspect of the short-term memory deficit is responsible for blocking the induction of long-term memory in these mutants? Is it that reversal probability, reversal distance or that both behavioural measures must reach a certain level of habituation before long-term memory can be induced? The *cmk-1* mutant data suggests altering both reversal probability and reversal distance habituation may be sufficient to block the induction of long-term memory, but is it necessary for both measures to be altered to block this induction?

To successfully induce long-term habituation worms are tested for memory when they are 120 hrs-old. Attempts to observe the expression of long-term habituation in younger adults (e.g. 96 hr-old worms) have been unsuccessful (unpublished data from our lab) – what differences are responsible for this? In chapter four, a comparison of responsiveness to the initial tap between these two age groups reveals no significant differences in either reversal distance or response

probability. A comparison of responsiveness to the final tap in a habituation series between these two age groups also revealed no significant differences in reversal distance. In contrast, habituation of response probability occurs more quickly in 120 hr-old worms than in 96 hr-old worms – although not to a significantly deeper level at the final tap in the presence of food, but this difference is significant if worms are tested in the absence of food. Perhaps the shallower habituation level of 96 hr-old worms masks the expression the long-term memory.

More evidence that habituation of response probability is more critical than habituation of reversal distance comes from a meta-analysis of long-term habituation studies performed in our laboratory from 2000-2011 by S. Nijeboer (unpublished data). Between 2000-2006, most of the habituation studies in our lab were performed by training animals as a group, transferring them to individual plates and then testing them for memory one worm at a time on a Petri plate (single-worm method; Rose et al., 2002). This time consuming testing method was altered in 2006 by testing worms in groups of 15-20 worms per Petri plate to expedite the process (multi-worm method; Butterfield, 2007). In the meta-analysis data from these two long-term memory training protocols were analyzed separately. Analysis of data from the single-worm protocol revealed that the significant difference between trained and untrained worms during the memory test, 24 hrs post-training, was due to a significant difference in response probability. In this data set there was no significant difference between trained and untrained worms for reversal distance. Analysis of the data from the multi-worm protocol revealed that the significant difference between trained and untrained worms during the memory test was due to significant differences in both response probability and reversal distance. This difference between the two protocols may be due to differences in worms behaviour when they are in a group versus solitary or due to the increased number of worms analyzed using the multi-worm method (gives us more statistical power to detect smaller effects). Spaced training induced long-term changes in response probability in both the single- and multi-worm protocols, but only induced long-term changes in

the multi-worm protocol. This suggests that although habituation of reversal magnitude may play role in long-term habituation, habituation of response probability could be more important.

The evidence that supports the hypothesis that habituation of response probability is necessary for expression of long-term memory is indirect, as this hypothesis has not been directly tested. To test it one should try to test for long-term memory in 120 hr-old worms with a mutation that alters habituation of response probability but not habituation of reversal distance (such as in *ncs-1(qa406)* and *F26F2.1(ok2626)* mutants) and vice-versa (such as in *hda-4(ok518)* mutants). This would allow one to directly test the role of each behavioural metric in the expression of long-term habituation.

5.3 Age-dependent changes in learning and memory begins in early adulthood

In Chapter Four, I report that *C. elegans* exhibit age-related changes in learning in memory that begin early in and extend throughout their reproductive period. As *C. elegans* age they habituate more deeply to stimuli in the measure of response probability. This change is due to their waning ability to behaviourally discriminate tap stimuli of different intensities. For example, I showed that unlike younger worms, 120 hour-old worms (at the end of their reproductive period) only weakly alter their habituation in a stimulus intensity dependent manner (i.e. habituate more deeply to less intense stimuli and conversely, habituate less deeply to more intense stimuli). Thus, I demonstrated that at least in *C. elegans,* age-dependent changes in learning and memory begin in early adulthood.

Although there appears to be controversy in the literature about when cognitive decline begins in animals, including humans, there is some other evidence that it begins in early adulthood. Salthouse (2009) tested the behavioural performance on 12 cognitive tasks of > 2000 healthy human subjects whose ages ranged from 18-60 years. The 12 cognitive tasks consisted of 3 different tasks for each of the following categories: reasoning, spatial visualization, episodic

memory and processing speed. He found that the 22-27 year old age group performed best on these tasks and that the next age group that was statistically different from the performance of this group was the 27-42 year old cohort. These results do not suggest that the ageing process is linear (subjects >60 years old showed an accelerated decline on the same tasks compared to this younger group of adults), but provide strong evidence that, similar to *C. elegans,* at least the behavioural effects of ageing begins in humans while they are still in their reproductive period. These behavioural findings are also supported by findings that a number of neurobiological variables that have been observed exhibit age-associated declines beginning in adults as young as 20 years old (brain volume & cortical thickness (Magnotta et al., 1999; Sowell et al., 2003; Allen et al., 2005; Fotenos et al., 2005; Salat et al., 2005; Kruggel, 2006; Pieperhoff et al., 2008), myelin integrity (Sullivan and Pfefferbaum, 2006; Hsu et al., 2008), neuromodulator receptor binding (Volkow et al., 2000; Sheline et al., 2002; Erixon-Lindroth et al., 2005), accumulation of neurofibrillary tangles (Del Tredici and Braak, 2008), and brain metabolites concentrations (Kadota et al., 2001)).

Why might ageing of the nervous system and behavioural cognitive decline begin during the reproductive period? Fitness is defined as the number of reproducing offspring in the next generation, and thus natural selection should only act upon those adaptations that benefit or hinder an organism's fitness potential. If cognitive deficits affect fitness then an evolutionary argument that could potentially explain these phenomena is that once an animal produces offspring there is no longer selective pressure on that animal for survival. As you start to reproduce, you increase your fitness and consequently selection pressure for survival decreases. In *C. elegans* we begin to see age-related changes in habituation in worms 84 hrs-old; by this age worms have already laid over 75 eggs (Lindemans et al., 2009; approximately 25% of their reproductive potential in the lab). This argument seems like a reasonable explanation for animals that either provides no parental care (such as *C. elegans*) or hatches/gives birth to precocious

offspring, but at first glance is harder to imagine how this may work in species that hatch/give birth to altricial young and require parental care (e.g. humans). Salthouse (2009) showed that age-related changes in cognition begins in humans aged 20-30 years old: a youthful age in the eyes of our modern society as the average age of Canadian mothers who gave birth in 2008 was 29.3 years old (Statistics Canada, 2011) . But the average age of onset of puberty in women ranges from 11-16 years old – suggesting that the ancestors of modern day humans may have began reproducing at a much earlier age. If during our evolutionary past humans started reproducing soon after they became fertile then this would allow for 10-15 years of parental care of their first born child before the cognitive decline Salthouse (2009) observed would begin.

It may also be that selective pressure for survival may not be on/off phenomenon (i.e. once an animal passes its reproductive age and its requirement for parental care the ageing process can begin) but may be a more graded process that begins soon after reproduction starts. Alternatively, it might be possible that our tests are quite sensitive and detect very subtle differences in behaviour that might not have an impact on *C. elegans* survival in their natural environment: the types of changes we observe as worms age between 72-120 hrs might not alter their fitness (i.e. they are not maladaptive and thus would not be selected against).

What is the importance of determining when cognitive decline begins? Healthcare use is typically most intensive in the first and last years of life (Ariste, 2005). Helping seniors maintain a high level of cognitive function could help alleviate some of the more expensive costs of late life healthcare, and if we know that cognitive decline begins earlier in life than previously thought a potentially effective way to approach this problem would be to begin interventions/treatments at an earlier age.

5.4 Conclusion

The studies presented in this dissertation contribute to our understanding of the molecular mechanisms underlying learning and memory and help define the time period of when these processes begin to succumb to the deleterious effects of ageing. I have demonstrated 1) that similar to other forms of long-term memories, long-term memory of habituation also requires the transcription factor CREB; 2) that CaMK1 is important for learning; and 3) demonstrated that in the nematode, *C. elegans*, age-related changes in learning occur over the course of the reproductive period, much earlier than had previously been established.

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