A CIRCUIT ANALYSIS OF HABITUATION IN

CAENORHABDITIS ELEGANS

by

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Abstract

The tap withdrawal response of the nematode <u>Caenorhabditis elegans</u> supports several forms of behavioural plasticity, including habituation (Rankin, Beck, & Chiba, 1990). The relative simplicity of this organism, both in terms of its nervous system and its genetic tractability, suggest that it would be a fruitful model system within which to investigate the neural and molecular substrates of learning. This report examines the production of the tap withdrawal response and the plasticity exhibited by that response at the cellular/circuit level. The neural circuit that mediates the tap response was identified through a program of single-cell laser microsurgery. Further behavioural analyses determined some of the functional properties of the tap withdrawal circuit neurons, both in the production of the tap withdrawal response and in the generation of the plasticity that that response expresses. First, this work demonstrated that the response was not unitary, but rather was composed of two competing reflexive behaviours: forward locomotion in response to posterior mechanosensory input and backward locomotion in response to anterior mechanosensory input. An animal's response to given stimulus was determined by the relative degree to which each of these reflexes were recruited. Second, it was demonstrated that each constituent reflex habituated in the absence of the other, and that habituation of the intact response was a summation of these two processes. Third, a dynamic network simulation of the circuit was used to predict the sign or polarity of the synapses that constitute the circuit. Fourth, it was demonstrated that at least two independent interstimulus interval-dependent processes were recruited during habituation: One that affects habituation kinetics and one that affects recovery from habituation. Finally, an analysis of the effects of tap withdrawal response habituation on other non-mechanosensory withdrawal behaviours was used, in conjunction with ablation studies, to identify potential loci of change within the circuit that

might underlie habituation. The implications both of the functional properties of tap withdrawal

circuit elements during habituation and the restriction of potential sites of change are discussed.

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Preface

The experiments reported in this paper have been published in a series of related papers. Experiment 1 was published in 1995 (Wicks & Rankin, 1995b); Experiments 3 and 4 are in press (Wicks & Rankin, in press-a, b). Experiment 2 is also published (Wicks, Roehrig & Rankin, 1996), but a significant contribution to the paper was made by Chris Roehrig. The bulk of these contributions have been placed in Appendix 1, and their inclusion in this document is for reference only and does not indicate intellectual proprietorship on my part. Experiment 5 is being prepared for submission as of the time of this writing.

I acknowledge that the above statement of authorship is accurate.

Catharine H. Rankin, Ph. D.

Acknowledgments

First, I would like to acknowledge my supervisor, Dr. Cathy Rankin, who was the living incarnation of Hume's advice to "be thee a philosopher, but amidst all your philosophy, be still a man". She never let me forget that the data aren't everything. Over the course of my doctoral studies a host of good friends have either acted as paragons of academic excellence that I have tried to emulate, or have made mistakes that I vicariously have learned from. These people include Rob Willson, Dave Mumby, Emma Wood, Chris Beck, Brett Broster, Bruce Hutcheon, Jeff Tomlinson, and Katherine Barrett; all of them have provided stimulating conversation, academic and otherwise, friendship and support at one time or another.

Introduction

In the attempt to understand the nature of the neural underpinnings of a phenomenon as complex as learning and memory, it has proven useful to make a number of simplifying assumptions. One major assumption often made by researchers is that the array of mechanisms that underlie learning are conserved across phylogeny (Byrne, 1987; Carew & Sahley, 1986; Quinn, 1984). The use of a simple system, while attempting to define what molecular and cellular events are required for the modulation of behaviour as a consequence of experience, eschews the intractable complexity that characterizes the central nervous system. Although this "phylogeny" assumption remains controversial, the high degree of conservation of those events and moieties that are thoroughly described in more than one species, suggests that the mechanisms that mediate plasticity are widely conserved (Arbas, Meinertzhagan & Shaw, 1991). To trace the chain of causality from the most basic biological events—gene expression, protein modification and enzymatic action—up through the cellular, circuit and systems levels of analysis to ultimately describe behaviour is the daunting task of the integrative neuroscientist. This task is made manageable with a system that is conspicuous for its simplicity at each of these levels. The work presented here represents an attempt to define the nature of the transition between the behavioural and circuit levels of analysis in the simple system, Caenorhabditis elegans. Specifically, this endeavor pursues three related lines of investigation: First, the neural circuitry that mediates a simple reflexive response is identified. Second, some functional aspects of the neurons within this identified circuit are suggested. Finally, the mechanisms by which the functional properties of neurons within this circuit might interact to modify the reflex as a consequence of repeated elicitation are explored.

<u>Simple circuits and the control of behaviour</u>

The adult <u>C. elegans</u> hermaphrodite possesses only 302 neurons (White, Southgate, Thomson & Brenner, 1986) . Given the limited size of its nervous system, the behavioural repertoire of this nematode is surprisingly diverse (see below for a survey of nematode neurobiology and behaviour). Nearly one-quarter of the nervous system (69 neurons) is dedicated to the production of locomotion, leaving only 233 neurons for the transduction of sensory input and the integration and production of complex behaviours. How so few neurons can control an organism that is both adaptive and behaviourally competent is a question that has significant ramifications for the control of artificial systems as well as our understanding of biological systems. <u>C. elegans</u> possesses the required combination of sophisticated behaviour and a sufficiently simple and well-defined nervous system to allow this question to be assailed.

Historically, the search for the neural substrates of reflexive responding was restricted to vertebrate systems (Sherrington, 1906). Although the use of the vertebrate system has met with undoubtable success (Cohen, Rossignol & Grillner, 1988), the relative simplicity of the invertebrate nervous system offers a number of advantages over the vertebrate nervous system. These advantages allow the description of behaviourally relevant neural events at or below the level of the single cell in a variety of preparations. Among the more dominant systems in this regard are the stomatogastric ganglion of both the spiny lobster (Selverston, Russel, Miller & King, 1976; Selverston, 1988) and the crab (Katz & Harris-Warrick, 1991; Weinmann, Meyrand & Marder, 1991), the <u>tritonia</u> escape circuit (Getting, 1981; Getting, 1983a; Getting, 1983b; Getting & Dekin, 1985), the control of heartbeat in the leech, <u>Hirudo</u> <u>medicinalis,</u> (Calabrese, 1979a; Calabrese, 1979b; Calabrese & Peterson, 1983), the leech local bend reflex (Lockery & Kristan, 1990a; Lockery & Kristan, 1990b; Lockery & Sejnowski, 1992), the control of locust flight (Pearson & Ramirez, 1990; Ramirez & Pearson, 1991), the proleg withdrawal reflex of

Manduca sexta (Jacobs & Weeks, 1990; Weeks & Jacobs, 1987), phonotaxis in flying crickets (Nolen & Hoy, 1986a; Nolen & Hoy, 1986b), and the crayfish tailflip response (Krasne, 1969; Wine & Krasne, 1972). Work in these, and other systems, has led to an understanding of some of the fundamental mechanisms by which small neural networks are capable of controlling behaviour (for reviews see (Getting, 1989; Harris-Warrick & Marder, 1991; Harris-Warrick, Marder, Selverston & Moulins, 1992)).

Among the more dominant concepts that have emerged over the last twenty years in this field are that of the command neuron (Kupfermann & Weiss, 1978; Wiersma & Ikeda, 1964), the central pattern generator (CPG) (Cohen et al., 1988; Harris-Warrick et al., 1992), the polymorphic circuit (Getting, 1989; Getting & Dekin, 1985) and distributed processing of information (Falk, Wu, Cohen & Tang, 1993; Kristan, Lockery & Lewis, 1995). These four concepts outline the range of the control of behaviour from the single neuron to the systems level. A command neuron represents one end of the continuum in conceptualizing the nature of the nervous control of behaviour; a command neuron is a single cell that is both sufficient and necessary for the production of a given behaviour under strictly controlled conditions. At the other end of the continuum is the recognition that some behaviours are the result of the integration of information in a distributed fashion, over large populations of neurons, such that no given neuron may be assigned a specific role in the production of that behaviour. Between these extremes lie, in order of increasing complexity, the CPG—a small network of neurons with specific intrinsic and synaptic properties that allow it to control motor behaviour in a temporally and spatially coherent manner—and the polymorphic circuit concept, which incorporates into the CPG the recognition that a single anatomically-defined population of neurons may control a battery of distinct behaviours, depending on the contextual or neuromodulatory state within which an animal exists.

Within simple circuits, a variety of mechanisms have been proposed to account for transient and rhythmic behaviours in both action potential generating (Getting, 1988) and graded, non-spiking (Roberts & Bush, 1981) systems. At the cellular level, properties of single neurons such as input resistance and spike frequency adaptation can influence circuit properties. In general, this control is achieved by varying the composition and compartmentalization of ionic currents found in the plasma membrane of neurons (Meech, 1979; Nobel, 1983). Equally significant are the properties of synapses between individual, or sets of, neurons which include, synaptic sign, strength, synaptic nature (i.e., electrical/chemical/neurohormonal), threshold (i.e., graded or dependent on spike invasion) and character (i.e., whether a synapse has single or multiple components of action) (Getting, 1983a; Getting, 1983b). Finally, the configuration of circuitry within an animal interacts with cellular and synaptic properties of that circuit's constituents to form a finite set of predominant circuit motifs which exercise control over behaviour. These motifs include recurrent inhibition, mutual excitation {e.g. Hebb's (1949) "reverberating circuit"}, feedback inhibition, and parallel excitation and inhibition (Getting, 1988; Getting & Dekin, 1985). The properties of a given circuit will be determined by interactions between all three sets of mechanisms, resulting in the potential for substantially complex activity, even in simple circuits. Bullock (1981) has argued that, even with an appreciation for this potential complexity, the existence of neuromodulators, novel transmission and transduction mechanisms, and other poorly understood variables should prompt caution in the conceptualization of "circuitry".

<u>Habituation and the modulation of behaviour</u>

Habituation, broadly defined, is a transient decrement in responding due to the repeated presentation of a stimulus (Hilgard & Marquis, 1940; Thompson & Spencer, 1966). This behavioural phenomenon has been recognized widely in a variety of preparations under various

names for nearly a century. Sherrington (1906), while describing the nature of reflexive responding in spinal cats, observed what Hilgard and Marquis (1940) cite as adaptation of the conditioned reflex. This terminology echoes that used by Pavlov (1927, pp. 48-49), who referred to the "rapid and more or less smoothly progressive weakening of the reflex to a conditioned stimulus which is repeated a number of times without reinforcement" as an internal inhibition of conditioned reflexes. Although Pavlov was referring to what is now called extinction, many early theories of habituation did not explicitly exclude cases of response decrement on the basis of properties of the eliciting stimulus, such as whether the subject had any previous experience with it. In contrast, Sokolov (1963), in a discussion of the "extinction" of the orienting reflex hypothesized that a "nervous model of the stimulus" was repeatedly compared to the stimulus applied to the animal. Responding to the stimulus was then dictated by the disparity between the model and the stimulus. In this case, although Sokolov was studying habituation, he referred to the phenomenon as extinction.

Early theoretical work in both the learning (Thompson & Spencer, 1966) and ethological (Thorpe, 1963) traditions reached similar conclusions regarding the nature of habituation. Consequently, several defining features of habituation have been described and were incorporated into developing theories of habituation (Hilgard & Marquis, 1940; Ratner, 1970; Sokolov, 1963; Thompson & Spencer, 1966). The observations about which consensus was reached included the following: decrement generally occurs exponentially as a function of stimulus number; the rate of decrement is dependent upon the interstimulus interval (ISI) applied; in the absence of stimulation, responding will recover; repeated bouts of habituation resulted in successively deeper and more rapid decrement; although decrement is primarily a pathway specific or stimulus-response (S-R) phenomenon, a generalization function can be constructed. In addition, although early accounts of response decrement emphasized active inhibitory constructs, it

became clear that excitatory constructs were required to explain observations both in the lab and in the field. These excitatory constructs were invoked to explain both decrement as the waning of an excitatory response—as opposed to the development of an inhibitory response prompted by Pavlov's early conceptualizations—and to explain facilitation in responding often observed under conditions when decrementing behaviour might have been expected.

In addition to the parametric features of habituation that were being identified as definitive, it was also recognized that habituation was nearly ubiquitous. A wide variety of behaviours in diverse organisms support this form of plasticity as evidenced by the rich ethological tradition which, very early, recognized the importance and prevalence of habituation in behaving systems (Hinde, 1970; Thorpe, 1963). Both Thorpe (1963) and Lorenz (1965) have suggested that habituation is probably the oldest mechanism by which behaviour might be modified by experience.

Even a cursory survey of the phylogenetic prevalence of habituation suggests that this might indeed be the case. Habituation has been observed in preparations as diverse as the infant heart-rate response (Clifton, Graham & Hatton, 1968), the arousal response of the goldfish Carassius auratus (Laming & McKinney, 1990), the escape response of the crab Chasmagnathus granulatus (Brunner & Maldonado, 1988; Lozada, Romano & Maldonado, 1990), the crayfish tailflip response (Krasne, 1969; Krasne & Teshiba, 1995), the proleg withdrawal reflex of the tobacco hornworm Manduca sexta (Wiel & Weeks, in press), foraging behaviour in the larval salamander Ambystoma punctatum (Pietsch & Schneider, 1990), the cleaning reflex of the fruit fly Drosophila melanogaster (Fois, Medioni & Le Bourg, 1991), the proboscis extension reflex in both the fruit fly (Corfas & Dudai, 1989) and the bee (Braun & Bicker, 1992), the nematode tap withdrawal response (Beck & Rankin, 1995; Rankin, Beck & Chiba, 1990; Wicks & Rankin, in press-a), the gill-siphon withdrawal reflex of Aplysia (Carew, Pinsker & Kandel, 1972;

Castellucci, Pinsker, Kupfermann & Kandel, 1970; Pinsker, Kupfermann, Castellucci & Kandel, 1970), and both physiological (Dworkin & Dworkin, 1990) and behavioural (Leaton & Supple, 1991) responses to auditory stimuli in the rat. Even the single-celled contractile protozoan Stentor, which lacks a conventional nervous system, demonstrates habituation of a mechanosensory contractile response (Wood, 1988a). Koshland and colleagues (Cheever & Koshland, 1992; Martin & Koshland, 1992), have even argued that the decreases in secretion of norepinephrine seen after repeated stimulation of the rat pheochromocytoma cell line (PC12 cells) is an example of habituation.

The confusion that arose regarding what constitutes habituation suggests the importance of placing habituation within the larger context of learning theory. Today, habituation is generally referred to as one example of a non-associative learning process; other examples include sensitization, the facilitation of a non-decremented response as a consequence of the presentation of a novel or noxious stimulus, and dishabituation, a similar effect observed with a decremented response (Groves & Thompson, 1970). This designation of "non-associative" is unfortunate for at least two reasons. First, since these phenomena are defined in terms of the absence of a key property of many forms of learning, they have been perceived at times as being less significant than associative forms of learning. This is surprising given the ubiquity and conservation of parametric features exhibited by some forms of non-associative learning across phylogeny (Hinde, 1970; Thorpe, 1963). In addition, since it appears that, at least in Aplysia, the mechanisms of classical conditioning appear to be an elaboration of hetreosynaptic facilitation— the mechanism that supposedly mediates behavioural sensitization—then further characterization of the mechanisms of associative learning might very well benefit from an understanding of habituation. Second, recently the concept of association-formation has come under critical scrutiny (Gallistel, 1990). Although associationism is still predominant in the literature, there is a

movement toward an understanding of what is being associated, that is, representations of stimuli and events, rather than the process of association-formation <u>per</u> <u>se</u>. Within this framework, a subject learns about predictive correlations between discrete stimuli, or the prevalence of an event within a given context, in the time domain, rather than forming associations between events without reference to time or event duration. This proposed paradigm shift (Kuhn, 1962) would thus dissolves the distinction between associative and non-associative forms of learning.

Although the distinction between associative and non-associative learning may be somewhat artificial, it is still worth describing as it may lend clues to how the two forms of plasticity may be related at a cellular level. Associative learning is based on the pairing of event occurrences. The roots of associationism in theories of mind can be traced back to Hume who wrote "It is evident that there is a principle of connection between the different thoughts or ideas of the mind, and that, in their appearance to the memory or imagination, they introduce each other with a certain degree of method and regularity." (Hume, 1748, reprinted 1955 p. 31). Hume listed three principles which bind ideas together, viz., "resemblance", "contiguity", and "cause or effect". D. O. Hebb, working within the Sherringtonian synaptic framework, provided one of the first, and certainly most robust, cellular formulations of associationism (Hebb, 1949). Hebb suggested that in order for an association to form at a neural level, it is necessary for both the presynaptic and postsynaptic elements to be active simultaneously. Thus, Hebb's rule of associativity incorporated Hume's concept of contiguity and was adopted as a unifying doctrine in several simple system models of learning (Berger, Laham & Thompson, 1976; Bliss & Lomo, 1973). Consequently, the search for the cellular correlates of associative forms of learning has taken the form of the search for potential sites of neuronal convergence of the conditioned stimulus (CS) and the unconditioned stimulus (US) pathways (Byrne, 1987).

Non-associative learning, on the other hand, is a consequence of the application of a single stimulus. It does not rely on pairing of events. In 1970, Groves and Thompson presented what has become the dominant theory of non-associative learning. In work with the acute spinal cat preparation, Spencer, Thompson and Neilson (1966c) demonstrated that dishabituation and sensitization were two expressions of one underlying mechanism, an idea originally suggested by Sharpless and Jasper (1956). This facilitating mechanism was distinct from that mechanism that mediated habituation. The behaviour of a subject in response to a given stimulus was conceptualized as the sum of these two distinct processes (Groves & Thompson, 1970). A key feature of this theory was that, although response facilitation was a "state" or system wide variable, response decrement was a pathway specific, "S-R" variable. Thus, it follows that the mechanisms underlying habituation, although not constrained by sites of stimulus convergence, might still be localized by their tendency to occur within the reflex arc.

This "dual-process" formulation is still the dominant framework for the study of non-associative learning. However, recent evidence has suggested that more than two processes may be present. For example, despite the fact that dishabituation and sensitization are formally similar, careful behavioural analysis suggests that they arise with different developmental timecourses in some simple preparations (Rankin & Carew, 1988) and that they may be affected in distinct ways by experimental manipulation (Marcus, Nolen, Rankin & Carew, 1988). This suggests that they are probably mediated by distinct mechanisms as well. Furthermore, Ratner (1970) and Edwards (1991) have both highlighted the importance of analyzing habituation within the context of an animal's ongoing behaviour, and that competition among multiple reflexes and behaviours may play a critical role in habituation of a given reflex. Ratner's response competition theory (1970) and Sokolov's (1963) stimulus comparator theory each suggest that the loci of habituation might not necessarily be localized within the reflex arc. Each of these theoretical

approaches has received some support from the analysis of habituation of the crayfish tailflip response (Krasne, 1969; Krasne & Teshiba, 1995; Wine & Krasne, 1972). In this system, habituation of a reflexive escape response appears to be largely due to the slow development of descending inhibition rather than the waning of an excitatory response. Thus, in this system, since habituation is a modulation of a reflex arc by higher centers, the locus of habituation is extrinsic to the reflex arc itself.

As the nature of habituation is to some extent system specific, any attempt to understand the mechanisms of habituation must be preceded by an understanding of the neural circuitry that mediates the behavioural response that is modulated by habituation. One can then determine where and how within the system plasticity is being expressed. The ideal system for the analysis of habituation would possess a complex behavioural repertoire, a dozen or so large labeled neurons connected by short electrically characterized processes. Furthermore, the organism would be amenable to the application of a variety of molecular genetic techniques. No such system exists. Therefore, investigators are faced with accepting compromise.

One approach, exemplified by the fruit fly Drosophila, investigates the molecular, rather than cellular, substrates of habituation using a primarily genetic strategy. Although this approach certainly has merits (Carew & Sahley, 1986; Dudai, 1988; Quinn, 1984; Tully, Preat, Boynton & Del Vecchio, 1994), the fruit fly shares with the vertebrate systems an array of disadvantages related to the complexity, inaccessibility and homogeneity of it's nervous system. An alternative and complementary approach selects systems (Aplysia, crayfish, etc.) on the basis of the large, accessible nature of many of their neurons and the presumed simplicity of neuronal interconnectivity.

Habituation within identified circuitry

Aplysia, the marine mollusk, has a respiratory organ, the gill, on its dorsal surface. The gill is protected by a fleshy mass called the mantle shelf. A mechanical stimulus applied to the siphon will cause a rapid contraction of the gill, the siphon and the mantle shelf. Repeated elicitation of this withdrawal reflex results in habituation of the behaviour that has both short-term (Pinsker et al., 1970) and long-term (Carew & Kandel, 1973; Carew et al., 1972) components. Furthermore, behavioural habituation of the mantle organs does not generalize to other sites, and is therefore pathway specific. This specificity allowed an analysis of the effects of a facilitating stimulus on both a decremented (dishabituation) and a non-decremented (sensitization) response (Carew, Castillucci & Kandel, 1971; Pinsker, Hening, Carew & Kandel, 1973). Since the circuitry controlling the contraction of the mantle organs had previously been described (Kandel & Tauc, 1965; Kupfermann & Kandel, 1968), this system proved to be one of the most promising for the study of the cellular correlates of non-associative learning.

The circuit that mediates the defensive gill-siphon withdrawal reflex in Aplysia consists of a relatively low number of identified neurons.{for a review see (Carew, 1984)}. It consists primarily of a battery of mechanosensory neurons (Byrne, Castellucci & Kandel, 1978; Castellucci et al., 1970) that make monosynaptic connections with the motor neurons that mediate the defensive withdrawal response (Castellucci et al., 1970; Kupfermann, Carew & Kandel, 1974). In addition to these monosynaptic connections, the sensory neurons in the circuit relay polysynaptic input via a set of heterogeneous interneurons, some of which have been identified (Hawkins, Castellucci & Kandel, 1981a).

In a series of experiments in which the cellular correlates of plasticity within this circuit were characterized, Kandel and colleagues were able to demonstrate that habituation of the defensive gill-withdrawal reflex was largely mediated by a decrease in synaptic drive at the

sensory-motor neuron interface (Castellucci et al., 1970; Kupfermann, Castellucci, Pinsker & Kandel, 1970). This was consistent with the results of Spencer et al. (1966a; 1966b; 1966c), who demonstrated that decrement of the electrophysiological correlates of habituation in the spinal cat were consistent with a decrease in synaptic efficacy. Subsequent work demonstrated that sensitization of the gill-siphon withdrawal reflex in Aplysia was produced by heterosynaptic facilitation of the same synapse that supported the habituation (Carew et al., 1971; Pinsker et al., 1973). This modulation of the identified synapse was mediated by polysynaptic interneuronal connections (Bailey, Hawkins, Chen & Kandel, 1981; Hawkins, 1981; Hawkins et al., 1981a; Hawkins, Castellucci & Kandel, 1981b). Furthermore, it was demonstrated that both short- and long-term components of the behavioural plasticity possessed correlates that were present at the same sets of synapses (Carew, Castellucci & Kandel, 1979; Castellucci, Carew & Kandel, 1978).

Having described the cellular correlates of non-associative learning, Kandel and colleagues were in a position to assail the molecular basis of habituation within an identified system. Specifically, an understanding of the locus of habituation suggested questions regarding the ways that the monosynaptic connection between the sensory and motor neurons could be modified to produce changes in behaviour. Castellucci and Kandel (1974) demonstrated that short-term habituation of the defensive gill-siphon withdrawal reflex in Aplysia was mediated by a decrease in the number of quanta of transmitter released at the sensory-motor neuron synapse. Subsequent studies by Klein, Shapiro and Kandel (1980) demonstrated that a decrease in the magnitude of an underlying calcium current was correlated with the observed decrease in neurotransmitter release. Gingrich and Byrne (1985) have developed a model of habituation, based on parametric studies of synaptic depression in the isolated ganglion (Byrne, 1982), that invokes both calcium current inactivation and a neurotransmitter depletion to explain homosyanptic depression.

The molecular correlates of sensitization were similarly studied. Castellucci and Kandel (1976) demonstrated that the cellular correlates of sensitization resulted in an increase in the number of quanta of neurotransmitter released at the sensory-motor neuron interface. The search for the mechanism by which this increase in transmitter release was effected revealed that there was also a modulation of a presynaptic calcium current involved. In this case, however, the modulation was 1) an upregulation of calcium influx into the presyanptic neuron, and 2) indirect, via the modulation of an inward potassium current. Klein and Kandel (1980) were able to demonstrate that direct electrical stimulation of the nerve connectives, or direct application of the putative facilitatory neurotransmitter serotonin, resulted in a decrease in efflux of potassium (the S-channel current). Serotonin was shown to have its effect via a defined intracellular elevation of cAMP and consequently, active protein kinase A. This then resulted in a broadening of the action potential and allowed greater influx of calcium with a consequent increase in neurotransmitter release.

Subsequent to the development of this model of heterosynaptic facilitation, Klein (1993) has argued that synaptic facilitation (and thus presumably its behavioural correlates) is not a unitary process, but rather that distinct mechanisms are invoked at rested versus depressed synapses. Neither action potential broadening (Hochner, Klein, Schacher & Kandel, 1986), nor the recruitment of the cAMP cascade (Klein, 1993) are required for the facilitation of a depressed synapse. At these synapses a recruitment of protein kinase C via the diacylglycerol pathway occurs (Braha et al., 1990), and plays a role in regulating transmitter release. This work largely confirms previous behavioural analyses of sensitization and dishabituation that suggested that the two processes were likely mediated by disparate mechanisms (Marcus et al., 1988; Rankin & Carew, 1987; Rankin & Carew, 1988).

Although the <u>Aplysia</u> system has proven to be very powerful—allowing meaningful transitions between the behavioural, cellular and molecular levels of analysis in pursuit of an alphabet of learning—a long ton of issues have been raised that cast doubt on the validity of several key assumptions made in this system with respect to the localization, and hence understanding of, learning. These criticisms take two forms: 1) demonstrations that the sensorimotor neuron synapse does not exclusively mediate either the reflex or the plasticity that the reflex supports and 2) demonstrations that changes in neurotransmission at that identified synapse may be independent of presyanptic ion flow.

Much of the work described so far assumes that changes at the monosynaptic connection between the gill-siphon sensory neurons and mantle shelf motor neurons mediates the defensive gill-withdrawal reflex. Initial demonstrations of the cellular correlates of the reflexive behaviour were studied either by direct stimulation of the mantle, or by electrical stimulation of the afferent nerve connectives (Castellucci et al., 1970; Kupfermann et al., 1970). In both these preparations, recordings in the motor neuron revealed that stimulation induced a complex excitatory post-synaptic potential (EPSP), with both monosynaptic and polysynaptic components. Subsequent studies concentrated on changes in the monosynaptic component of this EPSP, but evidence has accumulated that multiple sites of plasticity exist even within the <u>Aplysia</u> nervous system beyond that of the intensively analyzed monosynaptic connection. Specifically, it has been demonstrated that the complex EPSP (a reflection of the activity of all cells in the abdominal ganglia) is a better predictor of activity during the siphon withdrawal reflex than is the monosynaptic EPSP produced by the depolarization of the sensory neuron that was thought to mediate the behaviour (Wright, Marcus & Carew, 1991). One early estimate (40%) of the contribution of the polysynaptic component of the EPSP (Byrne et al., 1978) appears to have been conservative. A more recent report (Trudeau & Castellucci, 1992) of the relative

contributions of the mono- and poly-synaptic components of the EPSP, exploited a unique characteristic of gill-siphon withdrawal interneurons. Ionic manipulation of the extracellular medium allowed these interneurons to be effectively removed from the circuit by raising the action potential threshold of these cells to the point where they fail to fire in response to stimulation while the sensory neurons remain unaffected. Under these conditions, it was concluded that the polysynaptic component constituted more than 75% of the complex EPSP.

Early in the development of the model of non-associative learning in Aplysia, Peretz and colleagues (Peretz, 1970; Peretz, Jacklet & Lukowiak, 1976) demonstrated that the monosynaptic connection was not required to demonstrate either the reflex or modulation of the reflex as a consequence of experience; surgical excision of the abdominal ganglion was not sufficient to disrupt either the reflex or habituation of the reflex. Furthermore, if one postsynaptic motor neuron (L9) was tonically activated at low levels during habituation training, habituation of the reflex was prevented. Despite this however, the cellular correlate of habituation at the sensory neuron-L9 synapse (i.e., homosynaptic depression) was not affected by this procedure (Lukowiak, 1979). This dissociation of a behavioural modulation (habituation) and the presumed cellular correlates of that modulation highlighted the importance of the extensive, yet poorly characterized peripheral nervous system (PNS) in the mediation of these behavioural phenomena. Additionally, Lukowiak and colleagues (Leonard, Edstrom & Lukowiak, 1989) have carefully cataloged the array of gill movements evoked by mechanical stimulation. They conclude that the reflex is composed of at least four behavioural arrays, none of which require the ganglion that contains the monosynaptic connection between the sensory and motor neurons for expression.

A more recent study (Frost, Clark & Kandel, 1988) in which the synaptic changes between identified interneurons (Hawkins et al., 1981a) was carefully monitored during

sensitization training concluded that there were at least three sites of heterosynaptic modification within the gill-withdrawal reflex circuitry, in addition to the well-characterized sensorimotor neuron interface. Another report (Trudeau & Castellucci, 1993) confirms that there are multiple sites of change within the interneuronal network that can contribute to behavioural sensitization. This report extends the results of Frost et al. (1988) and also confirms that modulation of recurrent inhibition at these synapses (Fischer & Carew, 1993) is a powerful mechanism for the modulation of activity in the gill-siphon withdrawal circuit.

The distributed nature of the cellular correlates of both the reflexive response and modulation of that response was most clearly and directly highlighted by Cohen and colleagues (Falk et al., 1993; Zecevic et al., 1989). This group have taken advantage of the fact that the abdominal ganglion of Aplysia can be isolated without transecting the process tracts which connect it to the gill and mantel shelf. They bathed the ganglion in a voltage sensitive dye and optically, rather than physiologically, recorded the activity of hundreds of neurons within the ganglion simultaneously. The first report (Zecevic et al., 1989) from this group demonstrated that the activity of hundreds of neurons was altered during the elicitation of the gill-withdrawal reflex. Subsequently, Falk et al. (1993) systematically analysed how the activity of large numbers of individual neurons was altered as a consequence of habituation training. The results clearly show that the physiological changes associated with simple habituation training were widely distributed throughout the nervous system.

Another set of problems with which the Aplysia system has recently been beset is the disparity between the model (Hawkins et al., 1981b; Kandel & Schwartz, 1982) of how plasticity is expressed at the monosynaptic sensorimotor neuron interface and several novel findings. First, the model (Hawkins et al., 1981b) suggests that action potential broadening, accompanied by an increase in calcium influx underlies sensitization of the gill-siphon withdrawal

reflex. However, due to problems associated with the electrical isolation of the sensory neuron synapse from the soma (from which recordings are made), it has been difficult to demonstrate this directly by clamping the width of the action potential. Using a new culture technique that allows the control of presynaptic membrane voltage, Klein (1994) has demonstrated that eliminating the prolongation of the action potential during cellular facilitation training had no effect on synaptic augmentation. Furthermore, experimental broadening of the action potential during training had no effect on the degree of augmentation. Consistent with these results is work demonstrating that the only know calcium current that is modulated by 5-HT application does not appear to contribute to normal neurotransmitter release (Braha, Edmonds, Sacktor, Kandel & Klein, 1993; Edmonds, Klein, Dale & Kandel, 1990). Thus it appears that modulation of the calcium current at the presynaptic sensory neuron terminal plays little role in the augmentation of transmitter release at rested Aplysia synapses.

A second problem with the Aplysia model of synaptic plasticity has to do with the fact that, until recently, the mechanism of neurotransmitter release—including what the transmitter was—at the sensory-motor neuron interface was unknown. The model (Hawkins et al., 1981b) assumed that changes in synaptic efficacy associated with sensitization were due to presynaptic mechanisms. A recent series of papers suggest that neurotransmission at this synapse is mediated by an excitatory amino acid (Dale & Kandel, 1993; Lin & Glanzman, 1994a; Trudeau & Castillucci, 1993). Following these observations were several demonstrations of postsynaptic modification associated with sensitization. First, Lin and Glanzman (1994a; 1994b) have shown that the monosynaptic connection that presumably supports the changes that mediate both homosyanptic depression and heterosynaptic facilitation is capable of supporting long-term potentiation. Furthermore, Trudeau and Castellucci (1993) have demonstrated that there is an upregulation of excitatory amino acid receptors as a consequence of the induction of long-term

synaptic facilitation induced with 5-HT application. Thus it appears that any plasticity at the monosynaptic connection that is related to observed behavioural changes is due to the coordinated modulation of pre- and postsynaptic events.

Although the Aplysia system has faced a number of justified criticisms, it has greatly advanced out understanding of how non-associative plasticity is instantiated in a nervous system. Despite the fact that the role of distributed information processing in this system is just being recognized, it still appears that a substantial fraction of the behavioural modulation observed in the gill-siphon withdrawal reflex can still be attributed to changes at a single identified synapse. Furthermore plausible molecular mechanisms for these changes have been proposed. At this point however, the limitations of systems exemplified by Aplysia become clear. It is very difficult to confirm these molecular models in a system within which the control of genetic expression is not feasible. The fruit fly Drosophila represents an alternative system which allows the building and testing of molecular models via genetic lesion. Several concerns associated with this approach have been raised (Dudai, 1988) perhaps the most significant of which is that the mutants that are studied lack key enzymes throughout the animal's entire body at all stages of development. Thus, in the fruit fly, although discreet molecular questions can be asked, one can never be sure that the effect on behaviour is limited to a discreet portion of the nervous system. Furthermore the physiological and anatomical intractability of the fly nervous system limit the ultimate utility of this system. Drosophila and Aplysia are complementary systems within which compromise must be made. A prospective new model system, Caenorhabditis elegans, may represent a midpoint between the two extremes represented by the fruit fly and the sea slug; one that may be amenable to the application of techniques which previously have been restricted to use within only one system or the other.

Caenorhabditis elegans: Towards A Model System

The nematode worm C. elegans is an attractive candidate for a model system of learning and memory. First, it satisfies many of the requirements for a laboratory subject (Kenyon, 1988; Rankin et al., 1990). It is a small free-living non-parasitic soil nematode. It exists as an hermaphrodite, and this trait as well as its small genome, short reproductive lifespan, and it's cryoviability have made it a system of choice for genetic and developmental studies (Brenner, 1974). Consequently, a great deal of information has been accumulated on the way that this organism develops and interacts with its environment. A large genetic database has been established for use by the worm research community which includes a library of mutant strains, many of which have been extensively characterized (Waterson et al., 1992). Additionally, a number of molecular genetic and neurobiological techniques have been adapted for use with this organism including in situ hybridization (Albertson, 1984), integrative transformation (Fire, 1986), transposon tagging (Greenwald, 1985) and target-selected gene inactivation (Plasterk, 1992; Zwaal, Broeks, van Meurs, Groenen & Plasterk, 1993). Many of these techniques have already demonstrated utility in the dissection of behaviour (Gannon & Rankin, 1995)).

Furthermore the nematode has an extremely simple nervous system. The adult hermaphrodite worm has only 302 neurons, the connectivity of which have been defined to the synaptic level in individuals by serial-section electron microscopy (EM) (White et al., 1986). These observations have been repeated for various sections of the animal's anatomy including the anterior sensory anatomy (Ward, Thomson, White & Brenner, 1975; Ware, Clark, Crossland & Russel, 1975), the male tail (Sulston & White, 1980), and the posterior nervous system (Hall & Russell, 1991). From this work has emerged a number of principles concerning the conservation of connectivity between animals, as well as the nature of the connections within an animal (Hall & Russell, 1991).

A concern in any attempted circuit analysis is that the connections that mediate behaviour be unambiguously identified. In most current neurobiological systems this goal is unreachable (Peretz et al., 1976) due to the complex connectivity of even these "simple systems". Generally, the best that can be hoped for is that some of the functional connections can be isolated. In the worm system, the extremely simple process morphology and the serial-section EM data have allowed researchers to construct complete anatomical wiring diagrams of the entire nematode nervous system (with the exception of the pharyngeal nervous system (Avery & Horvitz, 1989)) and to use these diagrams to determine which of the anatomical connections are functional for a particular behaviour (Chalfie, Sulston, White, Southgate, Thomson, & Brenner, 1985; Nelson & Riddle, 1984; Wicks & Rankin, 1995a). When using this approach it is important to be aware of possible sources of functional connections which may lack obvious anatomical substrates {i.e., neurohumoral signaling (Selverston, 1988)}, nonspecific post-synaptic membranes (in the nematode, postsynaptic elements are not discernible, so synapses are defined presynaptically) or nonstandard transmitter systems such as nitric oxide which may not require an anatomically visible substrate for release (Bredt, Hwang & Snyder, 1990).

Another important trait that the nematode possesses is optical transparency. This allows for the visualization of every nucleus in the developing worm. Hence, a complete embryonic lineage is available for every cell in the larval animal (Sulston, Schierenberg, White & Thomson, 1983) as well as a complete post-embryonic lineage of every cell in the adult (Sulston & Horvitz, 1977). Additionally, this transparency allows for experimental manipulation of the nervous system. A laser microbeam can be focused down through the optics of a Nomarski microscope onto the nucleus of a specific cell. Intense or prolonged exposure to the laser microbeam results in the selective lesioning of the targeted cell (Bargmann & Avery, 1995; Sulston & White, 1980). This technique can be modified to elicit gene activation in single identified cells (Stringham &

Candido, 1993), manipulation of subcellular components (Hyman, 1989), or cytoplasmic fusion of adjacent cells (Schierenberg, 1984). Thus single-cell laser microsurgery has proven to be a valuable technique for the exploration of a wide range of cell-biological phenomenon, from behaviour to specification of cell fate.

The behavioural repertoire exhibited by C. elegans is surprisingly large considering the relative simplicity of its nervous system (the presumptive source of behaviour, however see (Avery & Horvitz, 1989)). In addition to locomotion (Croll, 1975), the worm is also capable of several forms of sensory transduction including mechanosensation (Chalfie & Sulston, 1981; Croll, 1975), chemosensation (Dusenbury, 1974; Dusenbury, 1980; Ward, 1973), thermosensation (Hedgecock & Russell, 1975), osmotaxis (Culotti & Russell, 1978), and perhaps photodetection (Burr, 1985). In addition the worm also displays several integrated behaviours, some surprisingly complex. The male exhibits a form of mating behaviour composed of several components (Hodgkin, 1983; Liu & Sternberg, 1995). Other studied behaviours include pharyngeal pumping (Avery & Horvitz, 1989), egg laying (Trent, Tsung & Horvitz, 1983), dauer induction (Albert, Brown & Riddle, 1981; Vowels & Thomas, 1992) and defecation (Liu & Thomas, 1994; Thomas, 1990). The pattern generators for many of these behaviours are being dissected by a variety of researchers using a combination of genetic and ablation techniques.

One of the best studied examples of circuit analysis in the worm is the delineation of the touch-withdrawal circuit (Chalfie et al., 1985). When an animal is touched gently on the tail with a hair, the animal accelerates forward; when an animal is touched gently on the head, it reverses. Ethologically, these responses are the expression of an escape strategy employed by the soil-dwelling worm to avoid danger. The neural circuitry underlying this response consists of six characteristic touch (or microtubule) cells (Chalfie & Sulston, 1981) (see Figure 1A), five pairs of

Figure 1: A) The positions of the six touch sensory neuron bodies are shown. Also included is the AVA interneuron cell body and full process: note the simple morphology (adapted form White et al (1986)). B) The touch withdrawal circuit (adapted from Chalfie et al (1985)). All cells are bilateral except AVM which is midline. Chemical connections = arrowheads; gap junctions = dotted line.

A

HEAD

PVM

TAIL

AVM and ALM
BACKWARD MOTION

PLM
FORWARD MOTION

B

REV

FWD

AVD

PVC

LUA

AVA

AVB

PLM

ALM

AVM
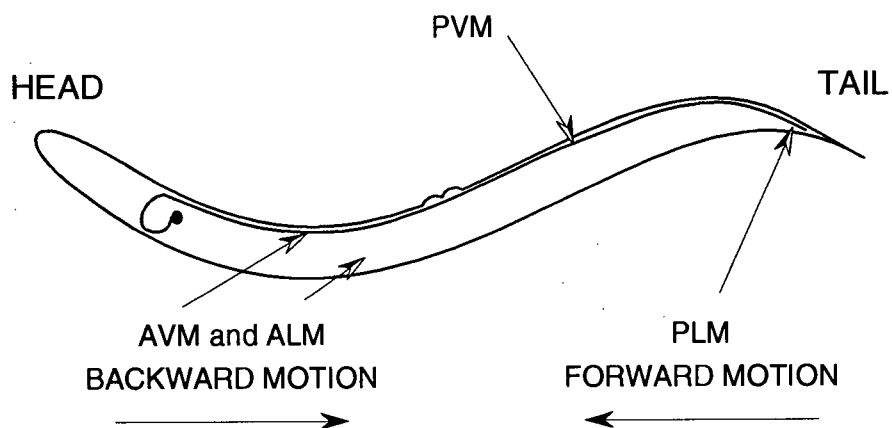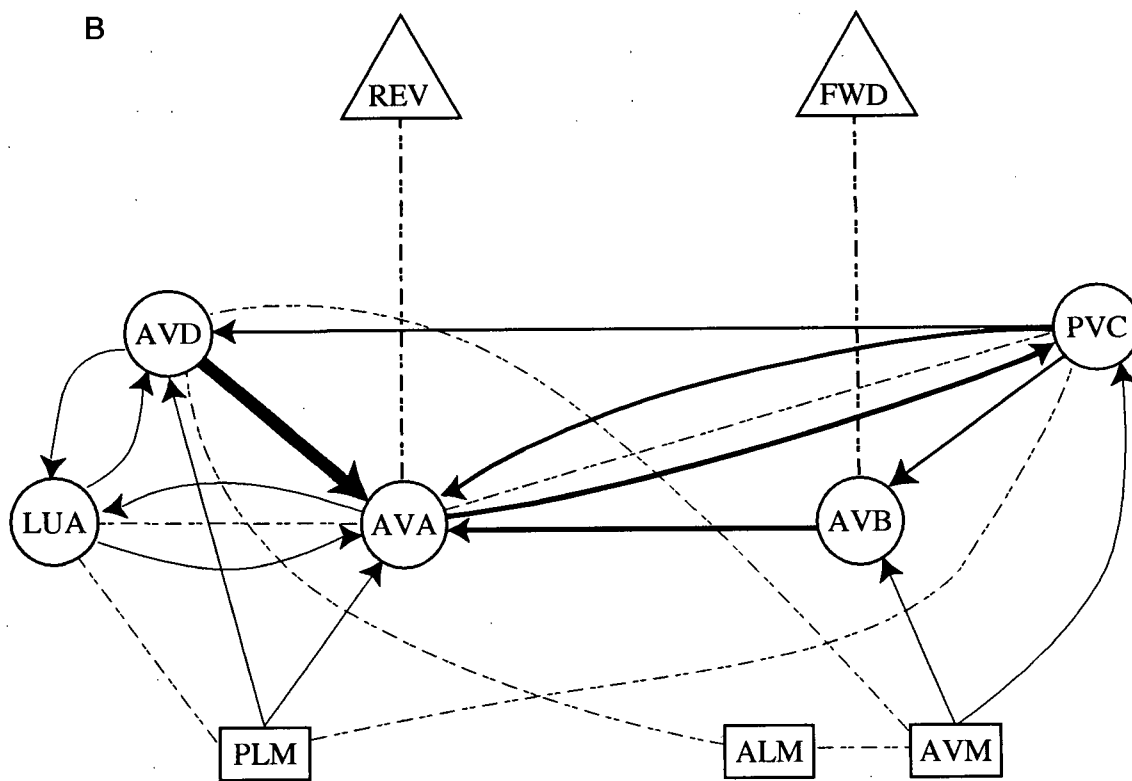
bilaterally symmetric interneurons, and sixty nine motor neurons assembled into three

homogeneous pools (85 total cells) {(Chalfie et al., 1985); see Figure 1B}.

Three techniques have been utilized to delineate the touch circuit. First, developmental

rewiring of the circuit was correlated with changes in the touch sensitivity of the animals

(specifically, the touch cell AVM, and the AS motor neuron pool arise from post-embryonic

lineages (Sulston & Horvitz, 1977), and do not hook into the circuit until later in larval

development). Second, ethylmethanesulfonate (EMS) mutagenesis was performed, and the

progeny of mutagenized worms were screened for touch sensitivity (Chalfie & Sulston, 1981).

The nature of many of the mutations that result in touch insensitivity has been described. Several

of the mutations are in proteins required for the function of the touch cells. The touch cells are a

group of six cells (AVM/PVM, PLM(x2), ALM(x2)) which contain a unique tubulin formation

consisting of a fifteen protofilament microtubule—other neurons in C. elegans contain tubulin

made up of 11 protofilament microtubules—and other associated structures (Chalfie & Thomson,

1982). The *mec-7* gene product codes for a beta-tubulin required for the production of this unique

motif (Savage et al., 1989). Other mutations which result in a loss of touch sensitivity are

products which specify cell fate or affect cell lineages (Chalfie & Au, 1989). For example, *mec-3*

is a homeobox containing gene that is required for the correct specification of the touch cells. In

the absence of *mec-3*, the touch cells appear to be more mundane neurons of unknown function

and lack the morphological features which otherwise characterize them (Way & Chalfie, 1988).

unc-86 on the other hand, is required for correct cell lineage specification and mutations in this

gene result in animals in which the touch cells are never formed (Chalfie & Sulston, 1981).

Another mutation of interest is the *deg-1* gene. This gene codes for a membrane bound protein,

alterations in which can impair the cell's ability to osmoregulate resulting in swelling and death of

several neurons, including the PVC interneuron of the touch circuit (Driscoll, 1992; Huang &

Chalfie, 1994). *deg-1* animals are tail-touch sensitive at hatching but loose this sensitivity by late in the second larval stage when the cell degeneration occurs (Chalfie et al., 1985). Several of these mutations have been shown to affect the touch and tap withdrawal responses (Rankin & Chalfie, 1989).

Finally, Chalfie and colleagues have investigated the role of individual neurons in the production of the touch response using the laser ablation technique (Chalfie et al., 1985). Of the touch cells, it was shown that: 1) PVM did not mediate any detectable response, 2) PLM's were required for tail-touch sensitivity, 3) ALM's were required for full head-touch sensitivity and 4) the AVM cell only mediated a partial response to head-touch (Chalfie & Sulston, 1981). Of the interneurons in the circuit; 1) AVB was required for forward movement but not touch sensitivity, 2) AVA was required for coordinated backward movement but did not mediate the touch sensitivity, 3) PVC was required for tail-touch sensitivity but did not mediate movement, and 4) AVD was required for head-touch sensitivity but was not required for movement (although some head-touch sensitivity was recovered in these animals after the AVM/AS cells made their connections) (Chalfie et al., 1985).

There are seven types of motor neuron in the animal that seem to mediate the locomotion associated with the touch response. Full ablation of any of these classes was not possible due to the massive cell death that would occur (Chalfie et al., 1985). The A motor neurons (12 VA's and 9 DA's) mediate backward motion. The B motor neurons (11 VB's and 7 DB's) mediate forward motion. These two classes of motor neuron are excitatory and probably contain a cholinergic neurotransmitter (Chalfie and White, 1988). The D motor neurons, unlike the other two classes, do not receive interneuronal connections, rather they receive connections from motor neurons on the other side of the body and play a putative inhibitory role for the inappropriate response and

are putative GABAergic neurons (Johnson & Stretton, 1987). Finally, the post-embryonic AS motor neurons appear to play a role much like the A motor neurons described above.

Although the excitatory and inhibitory roles played by the motor neurons described above is somewhat speculative (being based on behavioural observation, rather than electrophysiological evidence) these ideas have received support from the study of the larger nematode Ascaris suum. C. elegans, in addition to having extremely small neuron cell bodies (approximately 5 micrometers or less in diameter), possess a very durable cuticle which shatters glass micropipets upon penetration (recently, attempts have been made to deal with some of these problems and have met with some success, but problems still remain; see (Avery, Raizen & Lockery, 1995). Ascaris is much larger than C. elegans (it reaches 30 cm in length) and therefore has been amenable to electrophysiological recordings from motor neuron commisures to determine cell responses (Davis & Stretton, 1989a; Davis & Stretton, 1989b). Furthermore, the nervous systems of the two animals are highly homologous despite the superficial morphological differences. Indeed, the seven motor neuron classes described above from C. elegans have highly similar counterparts in Ascaris (Stretton et al., 1992). Recordings from the commisures of these motor neurons has confirmed the inhibitory role for the D motor neuron counterparts in Ascaris. These cells utilize GABA as a transmitter, and stimulation of these cells results in inhibition of muscle in a reduced preparation (Johnson & Stretton, 1987). Additionally, attempts have been made to model the nematode locomotion using assumptions based on these observations, and these models have met with some success (Erdös & Niebur, 1993; Niebur & Erdös, 1991; Niebur & Erdös, 1993).

That the nematode was capable of learning was not recognized until 1988 (Rankin & Chiba, 1988). Previously, the worm had demonstrated surprisingly complex behaviour including a presumptive association between an isotherm and the presence of food (Hedgecock & Russell,

1975). At the time, the implications of this observation were not fully recognized. In 1988, Rankin demonstrated habituation and dishabituation of the tap withdrawal reflex. Sensitization was also reported and, more importantly, long term (24 h) retention of habituation training (Rankin et al., 1990). Finally, the ability of the worm to demonstrate an associative form of conditioning has also been addressed. Preliminary data suggest that worms are capable of forming an association between the presence or absence of particular ions and a food source (the animals diet consists of the OP-50 strain of E. coli) (Kumar, Williams, Culotti & van der Kooy, 1989). Subsequently, the nematode has been used to explore several aspects of learning such as the effects of age on learning (Beck & Rankin, 1993) and the relationship between different forms of non-associative learning, such as dishabituation and sensitization (Rankin & Broster, 1992). Questions such as: to what extent do the various types of learning utilize similar underlying mechanism? and to what degree is learning localized? may be addressed using the worm.

The primary goal of the research proposed here is to gain some understanding of the nature of learning in an identified circuit in the worm. This project requires first that the neural circuit for the tap-withdrawal reflex, within which the plasticity occurs, be identified. The touch withdrawal circuit is a good starting point, but is in itself not sufficient. The cells that mediate the touch withdrawal response are not necessarily the same as those that mediate the tap withdrawal response and the roles of cells common to both responses may be different in each case. Touch and tap are distinct stimuli. The tap is diffuse and non-directed. It activates both the head- and tail-touch subcircuits simultaneously and therefor activates competing excitation and inhibition in the two subcircuits. The nature of this inhibition has been studied (Rankin, 1991; Wicks & Rankin, 1991). Touch, on the other hand is relatively intense and highly directed. The ethological significance as well as the neural substrates of the two stimuli are likely related but not identical. This may then be an example of a circuit that contains cells which function differently under sets

of slightly disparate conditions. This is the basis of the "polymorphic" circuit hypothesis that guides modern research (Getting & Dekin, 1985). The roles of the various touch circuit cells under these two conditions may then be different to the extent that the input to the circuitry produced by a tap stimulus is distinct from the input produced by the touch stimulus. The nature of these differences will be explored and the tap-withdrawal circuit defined. The next step in the analysis will be the determination of the role of cells in the production of habituation of the response to tap.

Another question that ablation studies may be able to address is: where in this circuit does habituation occur? There is substantial evidence from other invertebrate systems that habituation, or its physiological correlate homosynaptic depression, occurs in sensory neurons (Castellucci et al., 1970; Corfas & Dudai, 1989; Farley, Richards, Ling, Liman & Alkon, 1983; Kupfermann et al., 1970). In the touch withdrawal circuit there are three classes of sensory neurons—ALM, AVM and PLM—which transduce the touch stimulus. These cells each have unique patterns of connectivity, both in terms of excitation/inhibition and electrical/chemical connections and may each habituate differentially. One question that will be asked in the nematode preparation is whether different pathways in the circuit habituate at similar rates, or even whether all pathways habituate.

Summary

One of the major advantages of the "simple systems" approach to the study of learning and memory is that it allows the investigator to compensate for the inherent complexity of the phenomenon by utilizing a relatively well-understood nervous system. The nematode C. elegans offers two distinct investigative opportunities not found together elsewhere in a single organism: A fully delineated nervous system and a battery of molecular genetic techniques adapted for use in this organism. This research project is designed to assess the effects of cellular lesions in the

nervous system of <u>C. elegans</u> on the timecourse and morphology of one simple form of learning—habituation. First, neurons involved in the coherent production of one form of behaviour—the tap withdrawal response—will be identified. Then these cells will be ablated and the effects of these ablations on the production of the plasticity of interest will be assessed. In the future, it is hoped that these data will be used to direct studies in which molecular genetic lesions are used to form or confirm molecular models of habituation in the nematode preparation.

<u>Overview of Experiments</u>

In Experiment 1, laser ablation was used to identify circuitry that mediates transduction and integration of the mechanosensory input provided by the tap. In Experiment 2, a model of the tap withdrawal circuit was used to make predictions regarding some functional properties of tap withdrawal circuit neurons. Experiment 3 analysed the habituation of two antagonistic reflex responses that are elicited by a tap stimulus and suggested how the relative recruitment of these antagonistic behaviours might determine responding in the intact animal. In Experiment 4, laser ablation was used to alter the kinetics of habituation without changing the ISI used during training and thus establish whether the rate of recovery from habituation was a result of the same process that determined the kinetics of habituation. In Experiment 5 the loci of plasticity was restricted by analysing the effects of tap response habituation on other behaviours presumably mediated by subsets of tap withdrawal circuitry.

General Methods

Subjects and Materials

Cell Designations.

All cell classes are described using the classification of White et al. (1986). Unless

otherwise noted, all references to a particular cell class (e.g. ALM) refers to a pair of bilaterally

symmetric cells. Reference to a group of animals with one or more names of particular cell classes

followed by a negative sign (e.g. AVM,ALM-) indicates that all members of the indicated classes

were ablated in the group and that all other cell classes were left intact.

Subjects.

Unless otherwise noted, animals were originally obtained from the Caenorhabditis

Genetics Center and synchronously grown on Nematode Growth Medium agar seeded with E.

coli (OP50) as described by Brenner (Brenner, 1974). For laser studies, highly synchronous

animals were obtained by washing large numbers of eggs and adults in M9 buffer solution

followed by washing the animals in an alkaline hypochlorite solution (as described in Wood

(1988b) for the cleansing of infected colonies). The resulting solution was spun down in a table-

top centrifuge. The pellet was washed and resuspended in a drop of buffer and spread on an

unseeded plate. After 2-3 h, larval worms were collected for ablation.

Apparatus.

Laser pulses were delivered by a VSL-377 nitrogen laser (Laser Science, Inc., Cambridge,

MA). The beam was directed through a laser dye module (Laser Science, Inc., Cambridge, MA)

containing a Coumarin 440 dye (Laser Science, Inc., Cambridge, MA) which reemitted with a

peak gain of 437 nm. Single-cell ablations were performed under a 100x oil immersion lens

mounted on a Zeiss Axioskop equipped with Nomarski (differential interference contrast) optics

(Carl Zeiss Canada). The beam was directed down through the optics of the microscope with a semi-silvered mirror and targeted into the plane of optical focus with a beam expander (Laser Science Inc., Cambridge, MA).

All behavioural testing was done by observing worms on Petri plates filled with 10 ml of NGM agar, under a stereomicroscope (Wild M3Z, Leica Canada). All behaviour was recorded by a video camera (Panasonic Digital 5100) attached to a VCR (Panasonic AG1960) and monitor (NEC). A time-date generator (Panasonic WJ-810) was used to superimpose a digital stopwatch and time-date display on the video record. Taps (force of 1-2 N) were delivered to the side of the plate as described previously (Rankin, 1991). Figure 2 shows a schematic of the worm and the apparatus used during behavioural observations

Behavioural Analysis

Scoring of the tap response.

In response to tap, animals either reversed (swam backwards through some distance) or accelerated (swam forwards more rapidly), depending on their complement of cells (see Figure 3). Assistants, naive to the experimental condition, quantified response magnitude by tracing the path of the response using stop-frame video analysis onto acetate sheets. When reversals were elicited by tap stimuli, the magnitude of the reversal was assessed by tracing the length of the path described by the worm's backward locomotion. If a worm was already reversing when a stimulus was applied, then that datapoint was not used. When accelerations were elicited by a tap stimulus, the magnitude of the acceleration was assessed by subtracting the worm's velocity in the one second interval prior to the tap from its velocity one second after the tap. Velocity in these experiments was measured by determining the instantaneous velocity (in units of distance per second) of the animal prior to the tap as the distance through which the worm moved in a 1 s interval immediately prior to the tap and the instantaneous velocity of the animal (again, in units

Figure 2 Apparatus. A. Behavioural assessment was made by observing individual worms under a

microscope with attached video recording equipment. The timing of tap stimuli was controlled by

a Grass S-88 stimulator. B. The pulses delivered by the stimulator triggered an electromechanical

relay that rapidly deflected a copper rod. This rod thus tapped the mounted Petri plate upon

which a subject rested (A and B adapted from Beck, 1995). C. Laser pulses from a pulsed

nitrogen dye laser were delivered through the optics of a Nomarski microscope into the plane of

optic focus.

A

B

C
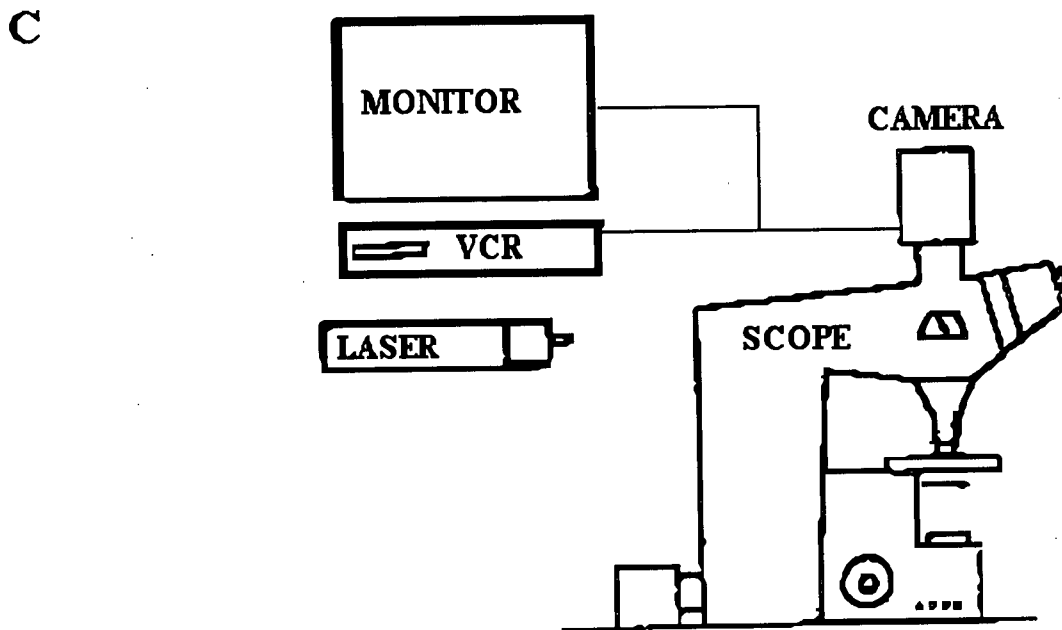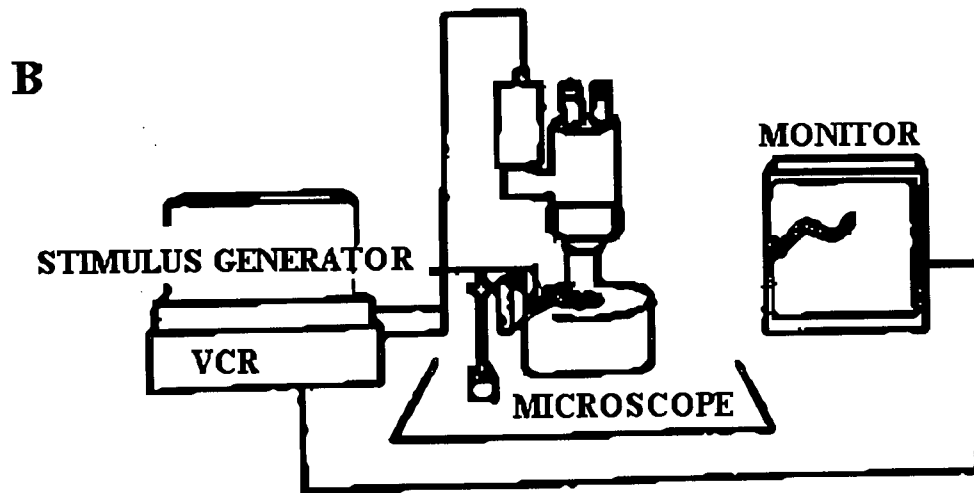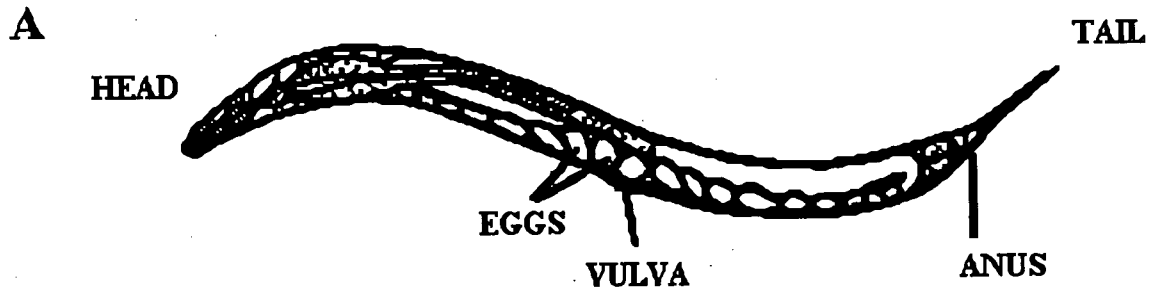
Figure 3 Response to Tap. Worms respond to a light vibrational stimulus either by reversing (A) through some distance and the resuming forward sinusoidal locomotion in a new direction or accelerating (B) along the pretap vector. The reversal measure is a distance (mm) described by the path through which the worm reverses and is indicated by the dark line. The time point which defines the end of the response (T = 1) is determined by the duration of the response itself. Reversals are often terminated when an animal enters into an "omega" waveform (Croll, 1975) in which the head and tail come into close proximity prior to the animal resuming forward locomotion. The acceleration measure (mm/sec^2) is defined by the difference in instantaneous velocities at T = 1 second and T = 0 second. These velocities (mm/sec) are defined as the distance that the worm travels (mm) over the one second intervals immediately prior to and following the tap.

A) REVERSAL

T=0 (TAP)

T=1

D

CHANGE IN HEADING

T=2

B) ACCELERATION

T=1

T=0 (TAP)

T=-1

V1

V2
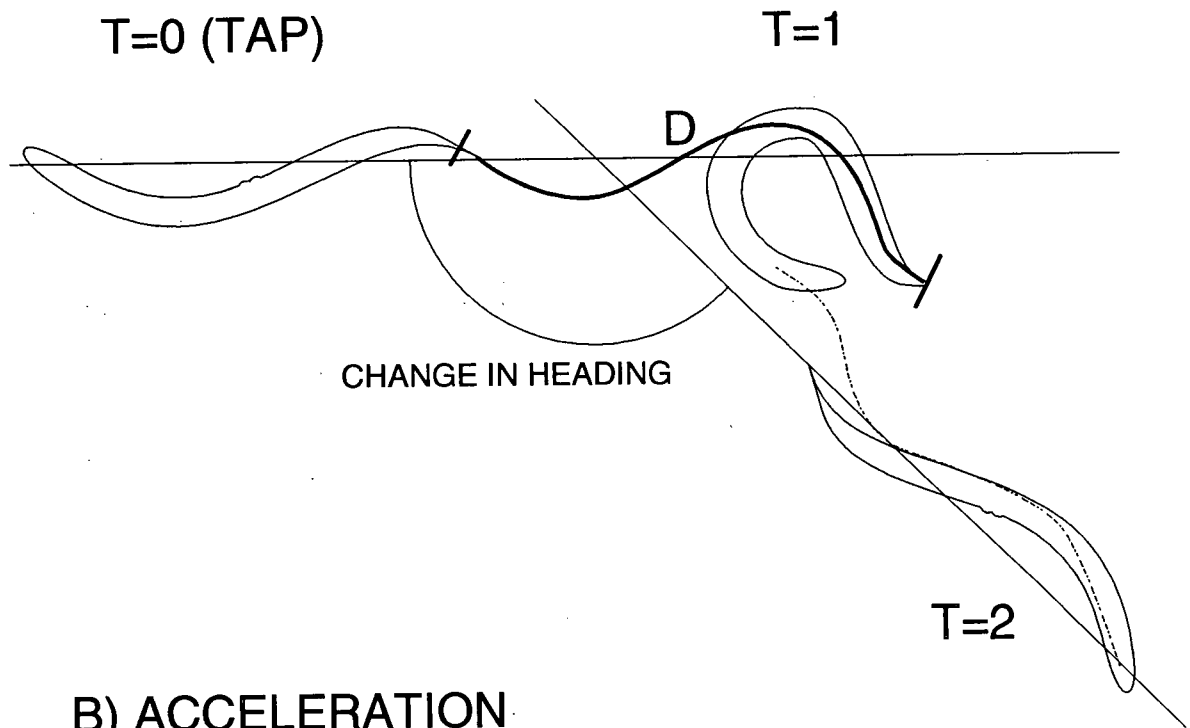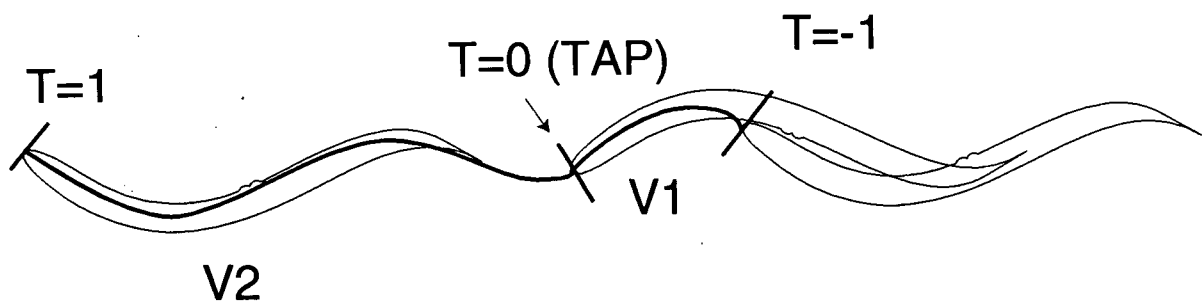
of distance per second) after the tap as the distance through which the worm moved in a 1 s interval immediately following the tap. Thus, the measure of a reversal response (a distance) and the measure of an acceleration response (a change in velocity) are incommensurate because they carry distinct units. The duration of the reversal is dictated by the nature of the animal's response; the acceleration measure is time-delimited by convention. The length of the traces were then digitized into machine readable form on a Macintosh computer using either a digitizing tablet (Summagraphics Bit Pad Plus) and Macmeasure software or a scanner (HP ScanJet 3c) and public domain NIH Image software (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov in /pub/nih-image).

Habituation, dishabituation, and recovery.

Habituation training was conducted as described in Rankin et al. (1990) with the following modifications. For experiments in which changes in the tap-withdrawal response magnitude was being assessed it was a high priority that animals not receive any mechanosensory stimulation prior to testing. Therefore animals were tested on the same plates as they were raised on. As a consequence, animals were tested in the presence of a bacterial lawn (E. coli). Individual plates were placed gently under the optics of the dissecting microscope and allowed to rest for 1 to 2 min. Then tap stimuli were delivered to the plates (at either 10 or 60 s intervals) and the behaviour was videotaped. Where appropriate, animals were dishabituated with a train of mild shock stimuli (6 60 V pulses at 10 Hz) delivered to the agar spanning the worm at the next scheduled tap as previously described (Gannon & Rankin, 1995). The worms were allowed to recover from the nonspecific effects of the shock for between 20 s and 30 s and then dishabituation was assessed with a series of three taps delivered at the same ISI as the training taps.

For those experiments that assessed recovery from habituation, animals were raised on individual plates seeded with E. coli, and transferred to unseeded testing plates just prior to testing. Individual plates were placed gently under the optics of the dissecting microscope and the worm was allowed to recover for 1 to 2 min. Then, tap stimuli were delivered to the plates (at either 10 or 60 s intervals) and the behaviour was recorded. Recovery stimuli were delivered to animals following intervals of 30 s, 5 min, 10 min and 20 min after the last habituation stimulus.

Laser Ablations

Single-cell laser ablations (Avery & Horvitz, 1987; Chalfie & Sulston, 1981; Sulston & White, 1980) were conducted by mounting highly synchronous animals (about ten at a time) in a small volume of sterile M9 buffer (less than one l microliter) on a wet agar pad containing approximately 10 mM sodium azide {an anesthetic, Wood, (1988b)}. Animals were covered with a 12 mm round glass coverslip sealed at the edges with Vaseline. Control animals were left under the microscope for approximately 45 minutes before being removed. Experimental animals were subjected to cellular ablations (bilateral where appropriate) before being recovered. The intensity of the laser beam was attenuated by interposing glass microscope slides between the laser and the microscope such that when the beam was focused in the plane of the coverslip it would just barely damage the glass coverslip (this intensity was ideal in that single laser pulses did little damage to a cell, but repeated pulses would destroy neurons). All damage was monitored visually. Any animals in which the damage was considered either incomplete or extra-neuronal, as well as any animals in which the targeted cell was not clearly identifiable were destroyed. Cell damage was monitored visually during the surgery and cell death was assessed using the criteria described in Bargmann and Avery (1995).

All cells were ablated in early L1, within 3 hr of hatching. All ablations were performed at the same stage in the development of the animal to control for the non-specific effects of

anesthesia, handling and food density on the testing plates. As a consequence, portions of the nervous system were still developing at the time of ablation. Some cells, derived from post-embryonic blast cells, were not yet present at the L1 larval stage. These cells included AVM (non-bilateral, ablated Q_R), PVM (non-bilateral, ablated Q_L), PHC/PVN/PLN (ablated T), and PVD/PDE (ablated V5). Thus AVM (non-bilateral), for example, was ablated in its precursor form by destroying the Q_R blast cell prior to cell division. All animals were recovered from the microscope slide and placed on individual agar plates seeded with OP50 E. coli within 1 hr of initial anesthesia and placed in a 20 degree C incubator.

Data Analysis.

Inferential statistics.

Most statistical comparisons of response magnitude were made using an analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) post-hoc tests (Statview, Abacus Concepts, 1992) where appropriate. Measures of response frequency were generally analysed with a Chi-squared statistic. Deviations from this are noted in each chapter where appropriate.

Two dependent measures were used to characterize the shape of a habituation curve: asymptotic level and initial rate. The asymptotic level of habituation was calculated by taking the mean percent-initial value of the responses to the last three tap stimuli during habituation. Dishabituation was assessed by subtracting the asymptotic response level of habituation from the mean of the first three responses to tap after the application of a shock. This measure was referred to as the dishabituation difference score. A one-sample t-test was then used to assess dishabituation for each group. Dishabituation was deemed present if this score was significantly above zero. Recovery was assessed with an ANOVA to compare recovery points to the asymptotic level of habituation.

<u>Descriptive statistics and habituation rate.</u>

As it was likely that the initial rate of habituation was related to initial response magnitude, and as we were concerned particularly with comparing rates of habituation between groups with significantly different initial levels of responding (Wicks & Rankin, 1995a), we standardized all data such that the mean of the initial response magnitude of each group was equal to 100%. Thus all habituation was expressed in terms of percent decrement from initial levels.

Curve-fitting was then used to determine the initial rate of habituation of each group in the following way. Groves and Thompson (1970) suggest that both a decrementing process and a facilitating process might underlie the net behaviour of an animal during habituation. Thus a curve of the general form

$$f(x) = Ae^{k1x} + Be^{k2x},$$ 1

where A, B, k1 and k2 are free parameters and x is the trial number, was used to produce best-fit lines for slope determination. A double exponential curve of this form can not be linearized; there is no unique solution which can be derived conveniently. Therefore, it was not possible to extract an initial rate parameter from the solution of this equation as has been done previously with solutions of single exponential equations (Glantz, 1979; Lukowiak & Peretz, 1980). Nevertheless, we elected to use a double exponential because habituation data are often characterized by two components—a facilitation process often observed early in the habituation series superimposed upon a decrementing process (Groves & Thompson, 1970). Furthermore, a visual inspection of some of the datasets reported here confirmed an initial facilitation component was evident and thus would require two exponential terms to be fit adequately.

We obtained values of the initial rate of habituation for each group by deriving a best-fit curve using a least-squares non-linear curve fitting algorithm (Microsoft Excel). The initial rate of habituation was estimated by calculating the initial slope of this function from its values on the first and second stimuli. Since the best-fit curve is smooth and continuous, this value was considered to be characteristic of the initial slope of the habituation curve for that group. However, since there was only one best-fit curve for each group, this approach failed to maintain any measure of variance in the dataset, making statistical group comparisons of initial slope problematic. To allow a quantitative analysis of slope, a best-fit curve and a corresponding initial habituation slope was determined separately for each animal. A mean-initial rate of habituation was then computed for each group and compared across groups using an ANOVA. Since the least-squares solution of an equation of the form given above is highly sensitive to the initial values of the four free parameters ($k1$, $k2$, A and B), we used the values of the parameters derived from the equation which represented the best-fit to the group data as starting points in the derivation of the individual-animal best-fit curves.

Experiment 1: Tap Withdrawal Circuit Determination

The neural circuitry that mediates the tap withdrawal reflex can be identified by ablating putative circuit cells and noting the effects of the ablation on the animal's withdrawal reflex. Once the role of a cell in the reflex has been established via laser ablation, other candidate cells can be identified on the basis of their connectivity. Chalfie et al. (1985) have identified the circuitry that mediates an animal's response to a light hand-delivered touch stimulus. This circuit was the starting point for the determination of the tap withdrawal circuitry. Since the nervous system consists of only 302 neurons, it should be possible to investigate the roles of all candidate neurons.

In this study we have defined the roles of the interneurons and sensory neurons of the touch withdrawal circuit in the production of the tap withdrawal response. In addition, results from the ablation of a number of neurons outside the touch circuit suggest a possible role for some of these cells in the tap withdrawal reflex.

Materials and Methods

Subjects

A total of 590 hermaphroditic C. elegans Bristol (N2) were used. In addition, 60 mutant animals (twenty each of *cat-1* (e1111)X , *cat-2* (e1112)II and the double mutant *cat-2*(e1112)II;*cat-1*(e1111)X) were analyzed.

Methods

For ablation studies, individual animals underwent laser microsugical removal of individual or multiple classes of neurons as described in the general methods, and the consequences of these lesions on the tap withdrawal response was assessed.

For the mutant studies, 4 d old mutant animals (as well as 20 N2 control animals) were isolated from synchronous colony plates and transferred to testing plates. Each animal was allowed to recover from the transfer for at least 1 min prior to the application of a single tap stimulus.

<u>Analysis</u>

Reversal magnitude data was analyzed by first expressing the length of all reversals that occurred in response to a single tap stimulus as a percentage of the individual worm's body length. This standardized measure was then compared across groups using a factorial ANOVA with Fisher's PLSD post-hoc tests (Abacus Concepts, 1992). Any animals that did not demonstrate a reversal to a single tap (less than 5% of the tested animals) were not included in the calculation of group means. If the animal paused in response to tap, its reversal magnitude was zero. Acceleration magnitudes were compared with a t-test on the mean of the first eight responses during habituation (a decrement in responding not due to motor fatigue or sensory adaptation). All frequency data were expressed as the fraction of worms reversing and analyzed with the Chi-squared statistic.

<u>Procedure</u>

During surgery, although extreme care was taken to ensure that all animals accepted for further experimentation received clean ablations, approximately 25% of the animals were remounted without anesthesia 2-3 hr after ablation, and checked to ensure that the target cell was destroyed. Although the target neuron(s) was destroyed in all of these animals, two worms were eliminated from the study due to initially undetected damage to adjacent cells.

Behavioural testing of ablation animals was done on the same plates on which the animals were isolated, shortly after the onset of egg laying at between 3 - 4 d post-hatching. Reversals were assessed by measuring the magnitude of each animal's response to a single tap. The

measurement of accelerations involved measuring the magnitudes of responses during trials with repeated stimulation to test for habituation. These animals received tap stimuli at a 10 s interstimulus interval (ISI). The mean of the first eight responses was calculated for each animal and used for comparisons.

A published report of these findings has been presented (Wicks & Rankin, 1995b).

Results

Experiment 1A: Effects of touch cell ablations.

Chalfie and Sulston (1981) described five specialized microtubule sensory neurons (see Figure 1B) which transduced the touch stimulus used in their experiments (a gentle touch applied with a hair to either the head or tail of the animal). These are divided into a bilateral pair of tail-touch cells (PLM), a bilateral pair of head-touch cells (ALM) and a single midline head-touch cell (AVM). A sixth midline microtubule cell (PVM) did not appear to play any significant role in the transduction of the touch stimulus (Chalfie & Sulston, 1981; Chalfie et al., 1985). These sensory neurons may transduce both the touch stimulus and the tap stimulus used in this analysis. The touch withdrawal circuit analysis may not, however, in itself be sufficient to describe the tap withdrawal reflex. The cells which mediate the touch withdrawal response are not necessarily the same as those which mediate the tap withdrawal response and the roles of cells common to both responses may be different. Although it has been noted that a tap stimulus does not elicit movement in touch insensitive mutants (Chalfie & Au, 1989; Chalfie & Sulston, 1981), touch and tap are distinct stimuli. The tap stimulus is diffuse and non-directed. It would presumably activate both the head- and tail-touch subcircuits simultaneously and therefore activate competing excitation and inhibition in the two subcircuits (Rankin, 1991; Wicks & Rankin, 1991). Touch, on the other hand is a relatively intense and highly directed stimulus. The ethological significance as well as the neural substrates of the two stimuli are likely related, but not identical. The tap

withdrawal response in the intact animal is sensitive to neural rewiring during development (Chiba & Rankin, 1990) whereas the touch withdrawal behaviour in the intact animal shows no developmental change, despite the observation that some of the cells in the touch circuit do arise post-embryonically. Additionally, response to tap has been more amenable to quantitative analysis than response to touch, because the tap is a repeatable, mechanically delivered stimulus, making it possible to reliably evaluate the magnitude of the withdrawal reflex. The measurement of response magnitude has proven to be a more sensitive measure of the relative roles of cells in this circuit than the frequency measure.

The first step in our analysis was to determine whether the tap stimulus was being transduced by the touch cells. Ablation of all five touch cells resulted in animals which generally did not respond to tap, however two animals did respond with very small reversals. The animals (n = 6) showed a significantly lower frequency (Chi-squared = 32.91, $p < 0.0001$, see Table 1A and Figure 4) of reversal response to tap, and the size of those reversals that occurred were significantly smaller than responses of control animals ($F = 5.48$, $p = 0.005$; Figure 5A). These results suggest that the tap stimulus is largely transduced by the five cells that Chalfie et al. (1985) described. However, there does appear to be some residual anterior input in the absence of these five cells which is sometimes sufficient to produce a reversal response. An electron microscopic reconstruction of the anterior sensory anatomy suggested that there were several cells in the tip of the head which could be mechanosensory in nature (Ward et al., 1975). Chalfie and Sulston (1981) noted some residual touch sensitivity in the tip of the head after ablation of the head-touch receptors (ALM and AVM). This observation was later confirmed and expanded upon by Kaplan and Horvitz (1993) who identified other mechanosensory receptors in the head by laser ablation which were distinct from the touch cells.

Figure 4: Changes in the frequency of response type as a consequence of ablation. This graph summarizes the effect of a variety of cellular ablations on the type of response elicited by a tap stimulus. Animals were scored either as not responding to a tap (gray bar), accelerating in response to tap (white bar) or reversing in response to tap (black bar). An asterisk beside the group name indicates a significant change in the percentage of reversals in response to a tap compared to control condition ("acceleration" and "no response" conditions were combined for this analysis). Note that PVD arises from the V5 lineage and that AVM arises from the QR lineage and that these two cells were removed by ablating the respective blast cell precursors.
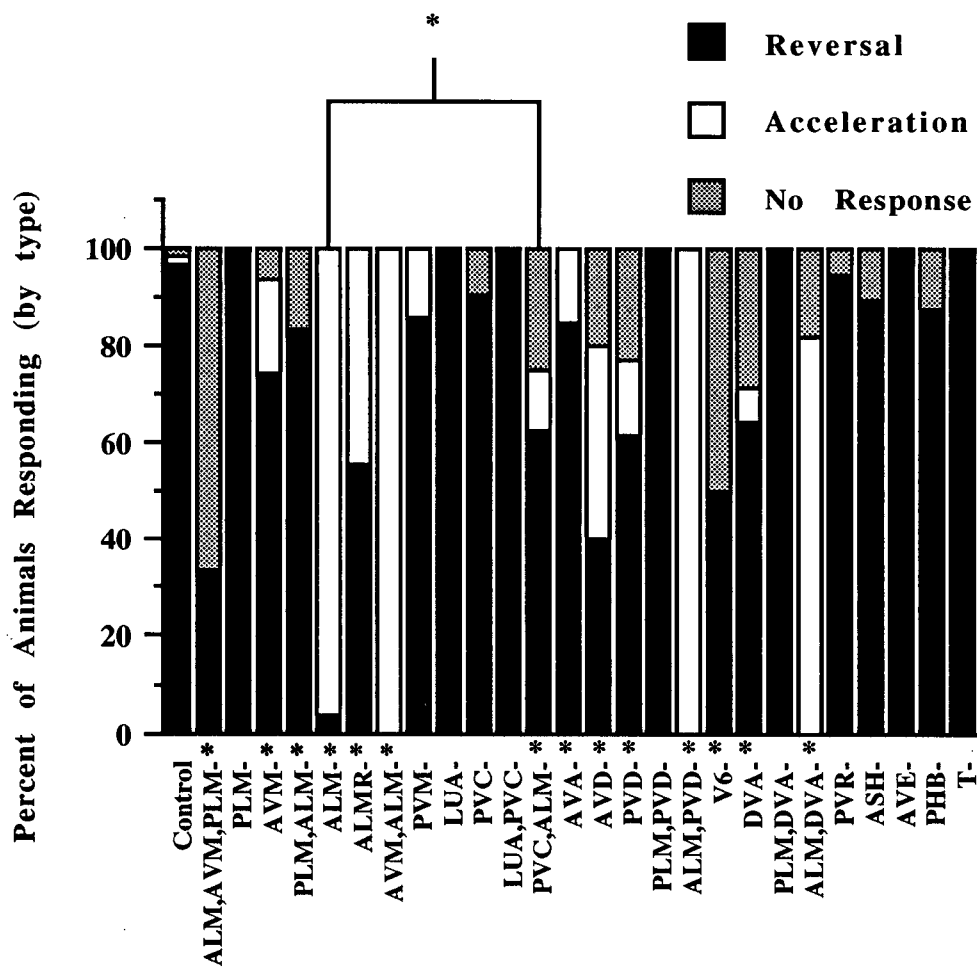
Figure 5: Changes in the tap withdrawal response magnitude as a consequence of ablation. The effects of cellular ablations on the magnitude of reversals (A) and accelerations (B) produced in response to a tap stimulus are shown standardized to the length of the worm. A) Significant differences in the reversal magnitude between an ablation group and the control group (far left bar) is indicated with an asterisks over that group. Two other comparisons were made (between PLM- and PLM,PVD- and between PLM- and PLM,DVA-)and are indicated by the bracketed lines. Of the cells not in the touch withdrawal circuit, only two are implicated in response to tap on the basis of the data presented here: PVD and DVA. B) The accelerations were measured by taking the mean of eight stimuli (see Methods and Figure 3). The addition of an AVM ablation to ALM- animals resulted in larger accelerations than ALM- alone, whereas the addition of either a PVD or a DVA ablation to ALM- animals resulted in smaller accelerations than ALM- alone. Note that PVD arises from the V5 lineage and that AVM arises from the Q_R lineage and that these two cells were removed by ablating the respective blast cell precursors. Error bars indicate the standard error of the mean. See Figure 3 for response measurement procedure.

A

Reversal Magnitude (y-axis)

Categories: Control, ALM,AVM,PLM-, PLM-, PLML-, PLMR-, AVM-, PLM,ALM-, PVM-, LUA-, PVC-, LUA,PVC-, PVC,ALM-, AVD-, V6-, PVD-, PLM,PVD-, DVA-, PLM,DVA-, PVR-, ASH-, AVE-, PHB-, T-

B

Acceleration Magnitude (y-axis)

Categories: AVM,ALM-, ALM-, ALM,PVD-, ALM,DVA-

Mean of Eight Responses
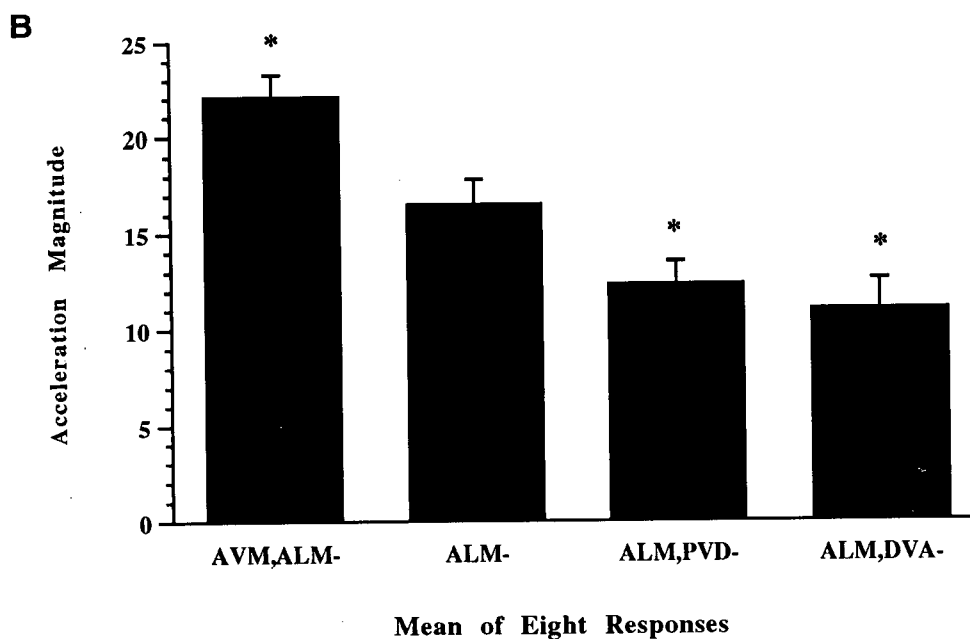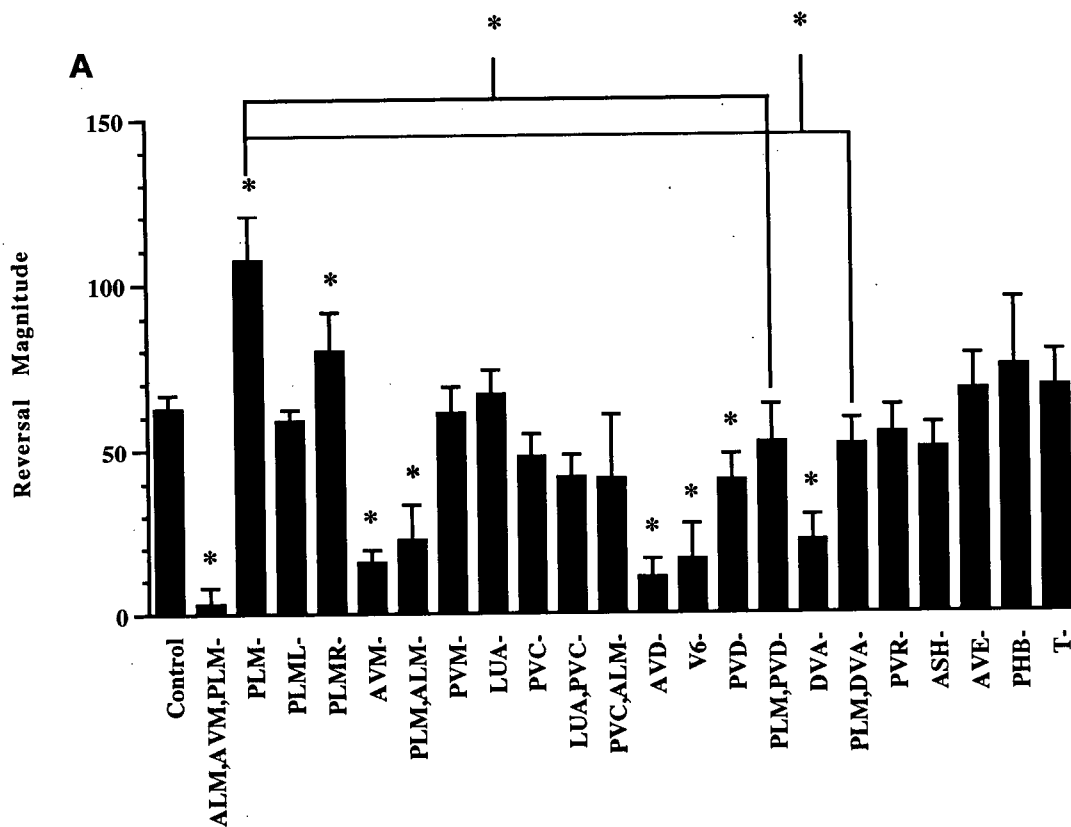
Animals in which the head-touch receptor ALM was bilaterally removed (n = 27) accelerated rather than reversed in response to the tap (Figure 4). Thus, in the absence of the ALM cells, the posterior input to the tap withdrawal circuit predominated and the animals accelerated forward. The ablation of the third remaining head-touch receptor alone (AVM, n = 29) resulted in a significant reduction in the frequency of reversal to tap (Chi-squared = 9.763, $\underline{p}$ = 0.002; Figure 4); AVM- animals occasionally accelerated rather than reversed. When AVM was ablated in addition to ALM in the same animal (n = 28), the animals, like ALM- animals, always accelerated in response to tap (Figure 4). Animals lacking the PLM cells (n = 35)—the only tail-touch receptors described by Chalfie and colleagues (1985)—always responded to a tap stimulus with a reversal.

An analysis of the response magnitude produced by animals lacking touch cells further clarified the roles of these cells. In the absence of the tail-touch cells (PLM), the reversals elicited by tap were significantly larger than control reversals (n = 16, $\underline{F}$ = 5.48, $\underline{p}$ < 0.0001; Figure 5A). The ablation of AVM also had a large effect on reversal magnitude, but in the opposite direction; AVM- animals reversed a shorter distance than did controls (n = 23, $\underline{F}$ = 5.48, $\underline{p}$ < 0.0001; Figure 5A). This effect may be due to the loss of gap junction input to ALM and AVD from AVM and/or the loss of putative inhibitory input (Chalfie et al., 1985) onto the AVB and PVC cells.

To further assess the role of AVM in the tap-withdrawal response, two additional ablations were performed. First, to determine whether AVM itself was capable of supporting a reversal response, the other two pairs of touch cells (PLM and ALM) were removed. Although animals in this group (PLM,ALM-, n = 8) responded with reversals to a single tap with a frequency equivalent to controls (Chi-squared = 0.135, $\underline{p}$ = n.s.), the magnitude of reversals produced was much smaller than control responses ($\underline{F}$ = 5.48, $\underline{p}$ = 0.0036; Figure 5A). Second, as reported above, ALM and AVM,ALM ablations resulted in animals which tended to accelerate

forward rather than reverse in response to tap. If AVM is actively inhibiting the posterior tap-response circuitry, the co-ablation of AVM and ALM in the same animal should produce larger accelerations than the ablation of ALM alone. However, the interpretation of the mean magnitude of acceleration to a single tap is complicated by a ceiling effect inherent in the measure used, as animals are already accelerating at near maximal levels to the tap stimulus. We therefore analyzed the magnitude of accelerations as the animals habituated, taking a mean acceleration of the first eight responses in a habituation protocol, reasoning that the measure of response magnitude in a decremented state may reveal group differences concealed by the ceiling effect. The mean magnitude of the first eight accelerations produced in response to tap stimuli delivered at a 10 s interstimulus interval (ISI) was significantly smaller for ALM- animals (n = 19) than for AVM,ALM- animals (n = 13) (F = 11.78, p = 0.0031; Figure 5B). A comparison of the rates of habituation of these two groups yielded no significant differences, thus the differences in mean magnitude were not the result of differences in the rate of habituation (using a within subject ANOVA, F = 0.821, p = n.s.). The detailed effects of ablation of tap withdrawal circuit elements on the reflex habituation dynamics will presented in detail elsewhere (Wicks and Rankin, in press-b, see Experiment 3).

Ablation of the PVM cell (n = 28) had no effect on either the magnitude (F = 5.48, p = n.s.; Figure 5A) or frequency (Chi-squared = 3.121, p = n.s.; Figure 4) of the tap withdrawal response. No evidence for its involvement in mechanosensory transduction could be identified. We also investigated whether we could demonstrate an effect of the ablation of a single PLM or single ALM cell. Chalfie and Sulston (1981) were unable to demonstrate an effect of removal of a single posterior sensory neuron (PLM) with a touch assay. The two PLM cells make asymmetric connections with the interneurons in the circuit and are not gap junctioned with each other. Both cells make gap junctions with other cells in the tap circuit, however only the right PLM sensory

Figure 6: Effects of unilateral tail-touch cell ablation on the tap withdrawal response magnitude.

The ablation of PLML had no effect on the reversal response magnitude, whereas the ablation of

PLMR resulted in a slight increase in reversal magnitude evoked by a tap stimulus. Removal of

LUA in addition to PLMR, had no further effect on the reversal magnitude elicited by tap. Error

bars indicate the standard error of the mean. See Figure 3 for response measurement procedure.
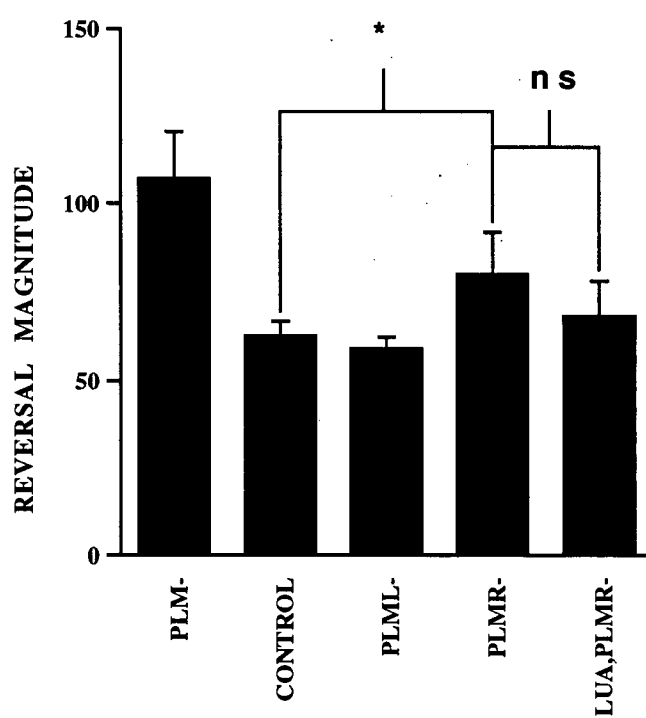
neuron makes chemical connections with the interneurons in the tap circuit (Achacoso & Yamamoto, 1992; White et al., 1986). Ablation of PLML (n = 7) had no effect on the magnitude of reversals produced (one tailed t-test, $\underline{t}$ = 0.337, $\underline{p}$ = n.s.; Figure 6). In contrast, the removal of PLMR (n = 13) resulted in animals which responded to tap with larger reversals than control animals (one tailed t-test, $\underline{t}$ = -1.718, $\underline{p}$ = 0.046; Figure 6). This result is consistent with the hypothesis of Chalfie et al. (1985) that the chemical connections from the sensory neurons are functionally inhibitory.

When we ablated a single ALM cell (n = 9) (we only ablated ALMR; the connectivity data do not suggest a functional asymmetry of the anterior touch cells) we found that the animal's pattern of behaviour was intermediate between the control pattern of reversals and the pattern of accelerations seen in bilateral ALM- animals. Of the nine ALMR- animals tested, five responded to a tap with a reversal and four animals accelerated (Chi-squared = 56.23, $\underline{p}$ < 0.0001). Thus there appears to be a limited form of redundancy inherent in the bilaterality of the anterior touch cells; a single ALM cell is capable of supporting a reversal response to tap, but does not do so as effectively as the bilateral pair of cells.

These results suggest that directed sensory input to the tap withdrawal circuit can be divided into three components: A posterior component completely mediated by the posterior touch cell class PLM, an anterior component carried by the two anterior touch cell classes ALM and AVM, and a small anterior component mediated by as yet unidentified cells. In general, removal of either PLM or ALM/AVM biases the animal such that its response is dominated by the other input. These antagonistic subcircuits may compete to produce the animal's behaviour. It is not the case that a lesion results necessarily in the reduction of a behaviour (indeed, PLM ablations result in an increase in response magnitude), but rather that behaviour is composed of a

number of competing subcomponents and an alteration in the circuitry mediating the behaviour alters which subcircuit is expressed in behaviour at any one time.

Experiment 1B: The effects of interneuronal ablations.

Based on Chalfie's analyses (Chalfie et al., 1985), the five pairs of interneurons described in the touch circuit can be placed into three classes. The first class of interneurons—consisting of the AVD interneurons in the head-touch subcircuit and the PVC interneurons in the tail-touch subcircuit—are not required for spontaneous locomotion. Rather, they are required for normal modulation of locomotion by head- and tail-touch respectively. In the absence of PVC, animals are tail-touch insensitive; in the absence of AVD, animals are head-touch insensitive (although Chalfie et al. (1985) recognized that animals do regain some head-touch sensitivity when the cell AVM makes functional synapses late in larval development). The second class of interneurons—consisting of the AVA interneurons in the head-touch subcircuit and the AVB interneurons in the tail-touch subcircuit—make electrical connections with the motor neurons required for backward and forward motion respectively (see Figure 1B). In the absence of either AVA or AVB, the animals are described as being backward- and forward-uncoordinated (Unc) respectively. That is, these two pairs of interneurons are required for normal spontaneous movement and have been described as command neurons. Thus the symmetry in the circuit evident at the level of the touch cells also seems to be expressed at the level of the interneurons. The third class of interneurons, the LUA cells, are described as connector cells which act to couple the PLM sensory neurons to the interneuronal level.

We systematically laser-ablated these five pairs of interneurons in an attempt to define their roles in response to a tap stimulus. The results of this experiment are summarized in Table 1B. In general, animals missing the class of driver cells (i.e., AVA and AVB) were studied only in terms of a description of the form of their tap withdrawal response. As such animals were

uncoordinated, any analysis of the magnitude of the withdrawal response would be difficult to interpret. A further limitation to these studies was that the two pairs of interneurons AVB and AVD are located adjacent to the pharynx deep in the lateral ganglia. Such a position makes it difficult to unambiguously identify and ablate these cells without damaging either the basement membrane of the pharynx or the adjacent neurons. Consequently, the number of animals in which these two cell pairs were confidently removed was limited (AVD, n = 4; AVB, n = 1).

Removal of the tail-touch modulators (PVC, n = 34) resulted in animals which consistently responded to a tap stimulus with reversals much like the PLM- animals described above. However, the magnitude of reversals produced by PVC- animals were not significantly different than control responses ($\underline{F}$ = 5.48, $\underline{p}$ = 0.066; Figure 5A). This ablation leaves the PLM cells and their chemical synaptic connections with the anterior mechanosensory circuit interneurons (AVD and AVA) intact. Chalfie et al. (1985) has suggested that these connections are functional and act to inhibit the anterior tap response circuitry much like the analogous connections from AVM discussed above act to inhibit the posterior tap response circuitry. This result provides support for this hypothesis.

However, the observation that the ablation of PVC—a neuron which is anatomically central to the touch circuit—leaves a functionally intact tap withdrawal response is paradoxical. Although the ablation of PVC might be expected to result in larger reversals due to the loss of putative excitatory input from the tail-touch sensory neurons (PLM), this ablation also disrupts complex recurrent connections in the circuit at the interneuronal level (see Figure 7). These two effects might counteract each other and result in a superficially normal tap withdrawal response in PVC- animals. To further explore our observations, we ablated PVC in ALM- animals. If PVC truly had no role in the integration of the tap withdrawal reflex, then the PVC,ALM- animals should accelerate to tap, much like the ALM- animals do. The PVC,ALM- animals (n = 8),

however, responded with significantly more reversals than did the ALM- animals (Chi-squared = 16.3, $p < 0.0001$, see Figure 4). Thus it appears that PVC does play a role in the integration of the tap withdrawal reflex.

Removal of the anterior AVB driver cells resulted in an animal which was forward-uncoordinated (Unc) as described by Chalfie et al. (1985), but appeared to respond to tap stimuli with normal reversals. Although this animal was capable of forward movement the form of this behaviour was disturbed. In general, the animal was able to propagate a waveform down approximately one-half of its body length. The tail of the animal was dragged along passively behind the animal as it moved. In response to tap, the worm reversed normally, with full coordinated involvement of the entire body length. The reversals often terminated with a slight kinking of the posterior body. Chalfie suggested that this residual ability to move forward was due to the presence of a complex sensory-motor network for control of head movements.

Removal of the posterior driver cells AVA resulted in animals which were backward-Unc, as described by Chalfie et al. (1985). This phenotype is the backward analog to the AVB-phenotype described above. That is, AVA- animals were incapable of producing a normal reversal but despite this, still attempted to respond to tap stimuli with reversals, although at a slightly lower frequency than control animals (n = 11, Chi-squared = 6.265, $p = 0.0123$; Figure 4). These reversals were abrupt, often resulting in the animal kinking up and freezing in response to tap.

Finally, ablation of the AVD cells (n = 4) resulted in animals which moved normally, but had a tendency to accelerate in response to a tap stimulus. Two animals produced very small reversals ($F = 5.48$, $p = 0.015$; Figure 5A), however this response type was less common than in wild type animals (Chi-squared = 19.608, $p < 0.0001$; Figure 4). This observation is consistent with the suggestion (Chalfie et al., 1985) that AVD acts as a functional connector between the

head-touch cells (AVM and ALM) and the backward locomotion driver cell (AVA); the ablation

of AVD attenuates the putative excitatory input to the motor neurons responsible for backward

motion.

When Chalfie et al.(1985) ablated the LUA cells they were unable to demonstrate any

change in the worm's touch sensitivity, although it was hypothesized that these cells might still

play a significant role as connector cells between PLM and the touch withdrawal circuit

interneurons by inhibiting the production of backward movement in response to tail-touch. This

hypothesis was well suited to testing using the quantitative tap assay. Removal of the LUA cells

had no effect on either the reversal frequency (n = 16, Chi-squared = 0.22, $\underline{p}$ = n.s.) or the

reversal magnitude ($\underline{F}$ = 5.48, $\underline{p}$ = n.s.) when compared to control animals (see Figure 4 and

Figure 5A). We also attempted to demonstrate a role for the LUA cells by ablating them in

animals that lacked the PVC cells, and thus much of the presumptive competing (forward

movement inducing) input to the circuit. Again, however, no effect of LUA ablation was

observed. The LUA,PVC- animals (n = 10) were indistinguishable from PVC- animals. Both

groups of animals consistently responded with reversals (Figure 4) of comparable magnitude ($\underline{F}$ =

5.48, $\underline{p}$ = n.s.; Figure 5A).

One further test of a role for LUA as a connector between the PLM cells and the

interneurons in this circuit was suggested by the connectivity data. The PLM cells make

asymmetric connections with the interneurons; PLML fails to make any direct chemical

connections with AVD or AVA, whereas PLMR makes several synapses with each. The left

PLM cell does, however, make indirect connections with AVA and AVD via the LUA cells. Thus

LUA may act as a connector only for the left tail-touch cell. To test this possibility we compared

PLMR- animals with LUA,PLMR- animals. These two groups each possess the left tail-touch

cell, however the LUA,PLMR- animals lack the LUA cells, and therefore also lack any direct or

indirect chemical synapses between PLMR and the AVA and AVD interneurons. The magnitude of reversals elicited in LUA,PLMR- animals were not significantly different from the PLMR- group (t-test, $t = 0.768$, p = n.s.; Figure 6). Thus the LUA cells do not appear to play a significant role in integration of the tap stimulus.

Experiment 1C: Ablation of cells outside the touch circuit.

Having delineated the roles of the touch circuit neurons in integrating the tap withdrawal response, we then attempted to define roles for any cells outside the touch withdrawal circuit that might also play a role in the response to tap. The results of these experiments are summarized in Table 1C.

Hypotheses concerning which cells might play significant roles in this response were formed on the basis of two sets of observations. First, a survey of the connectivity data provided by White et al. (1986) yielded many candidates. Any cells which made significant monosynaptic connections with known cells in the touch withdrawal circuit could play a role in the production of the tap withdrawal response. Second, literature concerning the roles of neurons in other behaviours (Bargmann, Hartwieg & Horvitz, 1993 for chemotaxis; Bargmann & Horvitz, 1991; Bargmann, Thomas & Horvitz, 1990; Kaplan & Horvitz, 1993 for head touch) was used to refine the list of candidates.

The cells investigated were PVR, PVD/PDE, ASH, PHB, AVE, DVA and the daughters of the T-blast lineage (PHC/PLN/PVN). The connections each of these cells make with identified cells of the touch withdrawal circuit are shown in Table 1C. Several of these cells have been tested for roles in mechanosensation (Kaplan & Horvitz, 1993; Way & Chalfie, 1989). Others stain for a marker of mechanosensory function (Siddiqui, Aamodt, Rastinejad & Culotti, 1989). Others make conspicuous connections with the touch circuitry and motor neurons required for movement. The reasons that each of these cells was considered are briefly outlined below.

The role of the PVD cells in the response to touch was assessed by Way and Chalfie (1989) with touch stimuli. It was thought that PVD may be a mechanoreceptor (Ward et al. 1975; Ed Hedgecock, cited in (Way & Chalfie, 1988). Way and Chalfie (1989) were able to show that PVD was required for sensitivity to "harsh touch". That is, in the absence of touch receptors, the animals would still react to a harsh touch stimulus (the worms were prodded with a platinum wire near their midbody region) by locomoting away from the stimulus (usually backwards); removal of the PVD cell attenuated this response. The PVD cell also expresses *mec-3* —a gene which controls the character of the six touch cells (Way & Chalfie, 1988)—and this mechanosensory function of PVD was absent in mutant *mec-3* animals (Way & Chalfie, 1989).

The PVD cells arise in a post-embryonic lineage as part of a pair of structures referred to as the post-deirids. Ablation of the V5 blast cells which give rise to these structures ensures that the PVD neurons are not formed. This procedure had a significant effect on the response to tap: The frequency of reversals in response to tap was significantly depressed as compared to control animals (n = 39, Chi-squared = 9.372, $\underline{p}$ = 0.0022; Figure 4), and the magnitude of those reversals which were produced was also reduced (n = 31, $\underline{F}$ = 5.48, $\underline{p}$ = 0.0143; Figure 5A).

One other pair of neurons (the PDE cells) was also ablated by this procedure and the effect of the ablation may have been mediated by the loss of these cells. However, the PDE cells make only sparse connections with the cells of the touch withdrawal circuit (PDE makes a total of 189 chemical synapses, only four of which are with members of the touch circuit), whereas the post-synaptic partners of the PVD cells are almost exclusively members of the touch withdrawal circuit (specifically, connections with PVC and AVA represent 110 of the 120 chemical synapses which PVD forms) (Achacoso & Yamamoto, 1992). Furthermore, the PDE cells are known to be dopaminergic and several mutants which have known defects in these neurons are available (Sulston & Horvitz, 1977). We tested three of these mutants (*cat-1*(e1111)X which lacks

dopamine in the processes, *cat-2*(e1112)II which has greatly reduced or absent dopamine, and the double mutant) on the tap withdrawal assay. No differences were noted in the magnitude or frequency of responding of either the *cat-1*, *cat-2* or the double *cat-2;cat-1* strains when compared to the N2 strain (data not shown). Thus the effect of V5 blast cell ablation on the tap withdrawal reflex appears to be independent of the normal functioning of the chemical connections from the PDE cells and is tentatively assigned to the loss of the PVD neurons. It has also been reported that the ablation of the V6 blast cell (the posterior neighbor of V5) affected the lineage specification of V5 such that the post-deirids were not formed; the V5 daughters go on to assume a hypodermal fate (Kenyon, Waring & Wrischnik, 1992). Ablation of the V6 blast cells in four animals had effects on the tap-withdrawal response that were consistent with this observation: Animals reversed less often than controls (Chi-squared = 15.05, p < 0.0001; Figure 4) and produced smaller reversals in response to tap than controls (F = 5.48, p = 0.013; Figure 5A). Furthermore, we ablated PVD in animals which also had the tail-touch receptors (PLM) ablated. These animals (n = 12) consistently reversed to tap, but the magnitude of the reversal produced by the PVD,PLM- animals was significantly smaller than those produced by PLM- animals (F = 5.48, p < 0.0001; Figure 5A).

There are two possible explanations for the attenuation of the reversal magnitude produced by PVD ablations. Either PVD—like AVM—biases the circuit toward reversals, in which case ablation of PVD would be expected to reduce the magnitude of the resultant reversal, or PVD acts to set the degree of excitability within the circuit by providing information to both the anterior and posterior portions of the circuit about the degree of background mechanosensory input. The connectivity of PVD suggests a role more in line with the second possibility: PVD makes approximately an equal number of connections with both anterior (AVA) and posterior (PVC) circuitry used in the touch response. These two hypotheses concerning the role played by

PVD produce different predictions about the effects of co-ablation of PVD and ALM. If the PVD cell biases the circuit toward reversals, then the predicted effect of co-ablation of PVD and ALM in the same animal would be to increase the acceleration magnitude. However, if PVD provides some level of excitation to the circuitry, then the mean acceleration magnitude of PVD,ALM- animals should be attenuated.

The results of tests with PVD,ALM- animals provides support for the hypothesis that PVD acts as a sensory neuron which sets excitability of the reflex circuitry, modulating the level of the animal's responsiveness. The mean acceleration magnitude of eight stimuli presented at a 10 s interstimulus interval of PVD,ALM- animals (n = 9) was significantly smaller than the mean magnitude of responses produced by ALM- (n = 19) animals ($\underline{F}$ = 11.78, $\underline{p}$ = 0.045; Figure 5B).

Some anterior circuitry not described by Chalfie et al. (1985) clearly has a significant role in the animal's movement and response to mechanical stimuli. None of the ablations described so far completely eliminate the animal's response to anterior touch, nor will any ablation completely destroy the animal's ability to move forward. As mentioned earlier, AVB ablations will produce animals which are unable to recruit the posterior body muscles in forward motion but are still capable of some rudimentary forward motion. Thus there must be some redundant function of the anterior sensorimotor circuitry.

The AVE cells are one of the three major interneuron sets which make connections with the more anterior motor neurons of the ventral nerve cord. Ablations of these cells (n = 7) had no detectable effect on the form of the animal's spontaneous movement. Furthermore, this ablation produced no significant change in the worm's response to a tap stimulus either in terms of the reversal frequency (Chi-squared = 0.113, $\underline{p}$ = n.s.; Figure 4) or reversal magnitude ($\underline{F}$ = 5.48, $\underline{p}$ = n.s.; Figure 5A). A possible interpretation of this result is that the function of AVE is entirely redundant with that of AVB in the intact animal, and that a role for AVE could only be

demonstrated in animals which lack both AVE and AVB. We were unable to obtain animals in which both of these interneurons were unambiguously co-ablated.

A number of other cells were selected as possible candidate members of the tap withdrawal circuit on the basis of their involvement in other related behaviours. For example, several cells have been implicated in the foraging behaviour and nose-touch sensitivity exhibited by the animal. Kaplan and Horvitz (1993) have studied the effects of various ablations on the animal's response to a light touch to the tip of its head. Among the cells which have been implicated in this behaviour via laser ablation is ASH. Ablation of ASH (n = 19) had no effect on either the frequency (Chi-squared = 3.100, $\underline{p}$ = n.s.; Figure 4) or magnitude ($\underline{F}$ = 5.48, $\underline{p}$ = n.s.; Figure 5A) of reversals.

A number of cells in the tail ganglia of the animal also make extensive connections with the cells so far discussed. Three of these (PHC, PVN, and PLN) arise from a single post-embryonic lineage (that of the $\underline{t}$ blast cell). The PHC cells have been reported to express touch cell-like markers in a specific mutant strain (Mitani, Du, Hall, Driscoll & Chalfie, 1993 citing Basson and Horvitz) and although this observation does not directly address the function of the PHC cells in wild type animals, it does suggest that PHC was worth investigating further. Ablation of the T-blast cell in early larval animals (2 hr post hatching, prior to the first $\underline{t}$ cell division) prevents all three of these cells from forming. This ablation (n = 6) had no significant effect on the tap withdrawal reflex (reversal frequency: Chi-squared = 0.085, $\underline{p}$ = ns; Figure 4; reversal magnitude: $\underline{F}$ = 5.48, $\underline{p}$ = ns; Figure 5A). Similarly, ablation of the PHB cells (n = 10), a pair of lumbar ganglion cells which makes connections with PVC and AVA, also had no effect on the tap withdrawal response (reversal frequency: Chi-squared = 2.902, $\underline{p}$ = n.s.; Figure 4; reversal magnitude: $\underline{F}$ = 5.48, $\underline{p}$ = n.s.; Figure 5A).

Another candidate cell in the tail ganglia is DVA. The single DVA cell has a process that synapses to both AVA and PVC (see Table 1C, Figure 7; the synaptic input from DVA to AVA is not represented on Figure 7 because this set of connections contains less than five members), the same cells to which PVD is also primarily presynaptic. Ablation of this single cell (n = 14) reduced the frequency of reversal in response to a tap stimulus (Chi-squared = 17.661, $\underline{p}$ < 0.0001). Furthermore the magnitude of the reversal response was attenuated as a result of this ablation ($\underline{F}$ = 5.48, $\underline{p}$ = 0.0003). In addition to the connections with the interneurons noted above, DVA also makes chemical synapses with some of the motor neurons which mediate forward movement.

Two additional ablations were performed in order to further explore the finding that DVA appears to play a role in integrating the tap withdrawal response. First, we ablated DVA in addition to PLM to determine if the removal of DVA would attenuate the reversal magnitude of the large reversal produced by the PLM ablation. These animals (PLM,DVA-, n = 9) reversed to tap, but the mean reversal magnitude of the responses was significantly smaller than the mean magnitude of PLM- animals ($\underline{F}$ = 5.48, $\underline{p}$ = 0.0002; Figure 5A). Second, we removed DVA in animals which also had ALM ablated. Thus we were able to analyze the effects of the DVA ablation on the magnitude of accelerations produced in response to tap. The accelerations produced by ALM,DVA- animals (n = 11) were significantly smaller than the accelerations produced by ALM- animals ($\underline{F}$ = 11.78, $\underline{p}$ = 0.0057; Figure 5B). This pattern of results, in which the magnitude of both reversals and accelerations was decreased, is similar to that obtained for the PVD ablation.

Finally, the cell PVR was ablated to determine if it had any role in producing a normal tap withdrawal response. The microtubule cells are a group of six cells (AVM, PVM, PLM(x2), ALM(x2)) which are genetically and biochemically distinct from other cells in the organism

Table 1: Summary of ablation results. The number of animals in each ablation group used to quantify the response to a tap stimulus, along with a summary of the results of those ablations for the sensory neurons (A) and the interneurons (B) of the touch circuit described by Chalfie et al. (Chalfie et al., 1985), as well as a number of additional neurons (C) not found in the touch circuit are shown. The relevant connectivity of the non-touch circuit neurons is also presented. (Abbreviations: n.a. = not applicable; con = control group; Unc = uncoordinated).

Table 1A Ablation	(n = ?)	Compared to...	Reversal Frequency	Response Magnitude	Notes
ALM,AVM,PLM	(n = 4)	con	decrease	decrease	Slightly sensitive to anterior mechanosensory input
PLM	(n = 16)	con	increase	increase	always reverses
PLML	(n = 7)	con	increase	no change	gap junctions only
PLMR	(n = 13)	con	increase	increase	makes all chemical connections
ALM	(n = 19)	con	decrease	na	always accelerates
ALMR	(n = 9)	con	decrease	na	accelerates half the time
AVM (Qr Blast)	(n = 18)	con	decrease	decrease	Q_R blast cell ablated
ALM,AVM	(n = 13)	con	decrease	na	always accelerates
		ALM	no change	increase	larger accelerations

Table 1B Ablation	(n = ?)	Compared to...	Reversal frequency	Response magnitude	Notes
LUA	(n = 16)	con	no change	no change	
LUA,PVC	(n = 10)	PVC	no change	no change	
LUA,PLMR	(n = 14)	PLMR	no change	no change	
PVC	(n = 20)	con	no change	no change	always reverses
PVC,ALM	(n = 6)	ALM	increase	na	
AVD	(n = 4)	con	decrease	decrease	
AVA	(n = 11)	con	decrease	na	Animal is backward Unc.
AVB	(n = 1)	con	no change	na	Animal is forward Unc.

Table 1C Ablation	(n = ?)	Compared to...	Reversal frequency	Response Magnitude	Synapses to...	Synapses with...
AVE	(n = 7)	con	no change	no change	AVA, AVB, PVC	AVA, AVB, ALM, PVC
PVD (V5 Blast)	(n = 31)	con	decrease	decrease	AVA, PVC	...
V6 Blast	(n = 5)	con	decrease	decrease		
PLM,PVD	(n = 12)	PLM	no change	decrease		
ALM,PVD	(n = 9)	ALM	no change	decrease		
ASH	(n = 18)	con	no change	no change	AVA, AVB, AVD	...
PHB	(n = 10)	con	no change	no change	AVA, AVD, PVC	...
PVM	(n = 28)	con	no change	no change	AVM, PVC	...
PLN (T blast)	(n = 6)	con	no change	no change	...	...
PHC (T blast)	(n = 6)	con	no change	no change	PVC, AVB, AVA	PVC, PLM
PVN (T blast)	(n = 6)	con	no change	no change	PVC, AVB, AVA, AVD	AVB
FLP	(n = 1)	con	no change	no change		
IL1,OLQ	(n = 1)	con	no change	no change		
DVA	(n = 14)	con	decrease	decrease	AVB, PVC, AVA	PVC, PLM
PLM,DVA	(n = 9)	PLM	no change	decrease		
ALM,DVA	(n = 11)	ALM	no change	decrease		
PVR	(n = 19)	con	no change	no change		PLM, ALM, AVM

Figure 7: The simplified nematode tap withdrawal circuit. The hypothesized circuit which

mediates the nematode tap withdrawal reflex consists of seven sensory neurons (squares), nine

interneurons (circles) and two motoneuron pools (not shown) which produce forward and

backward locomotion (triangles). All cells represent bilateral classes of cells except AVM and

DVA which are single cells. Chemical connections are indicated by arrows, with the number of

synaptic contacts being proportional to the width of the arrow. Gap junctions are indicated by

dotted lines. This circuit has been simplified for ease of presentation in two ways. First, the

bilateral symmetry of the circuit has been collapsed and second, only connections with an average

of greater than five synapses are shown.
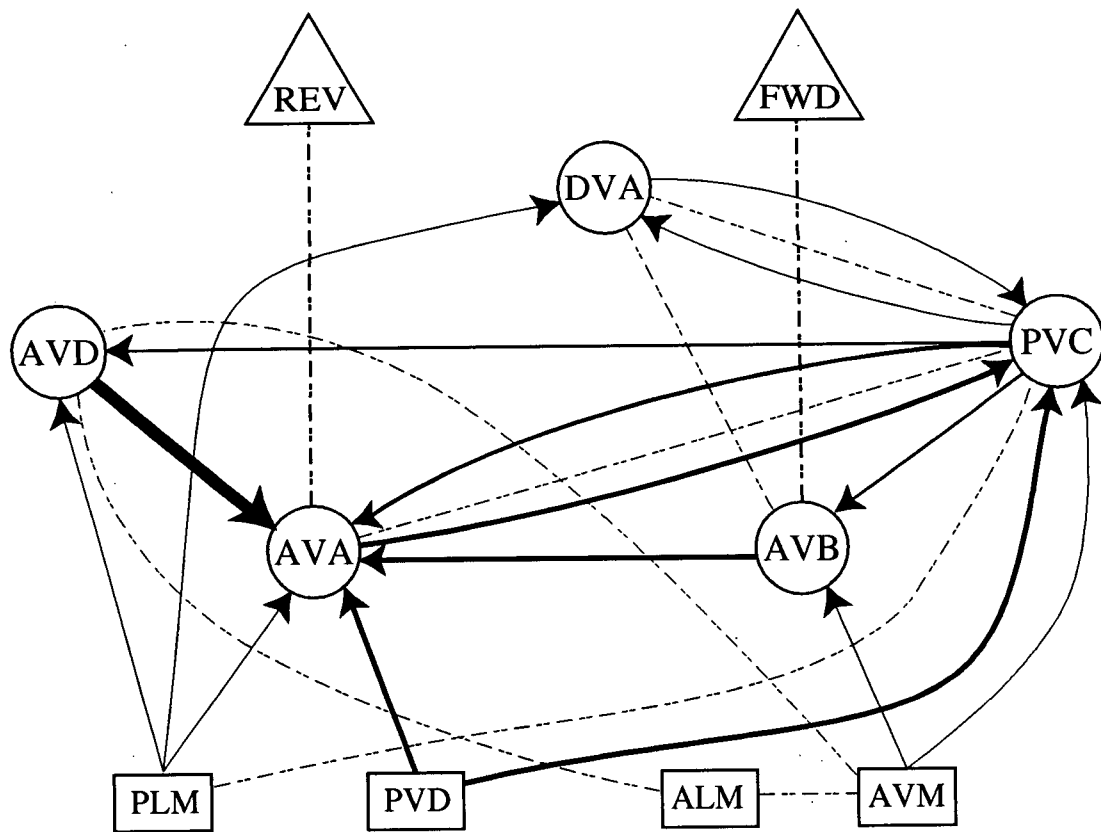
(Chalfie, 1993; Chalfie & Thomson, 1982; Savage et al., 1989). Consequently, markers exist which recognize these cells specifically. In particular, one microtubule antibody which uniquely stains the six cells mentioned above also stains the PVR cell (Siddiqui et al., 1989). Given that the PVR cell also make significant monosynaptic connections with the circuitry used in the touch response, it was tested for its role in the tap reflex. We performed post-embryonic PVR ablations (n = 19) and found no effect of this ablation on either the reversal frequency (Chi-squared = 0.756, $\underline{p}$ = n.s.) or reversal magnitude ($\underline{F}$ = 5.48, $\underline{p}$ = n.s.).

Discussion of Experiments 1A-1C

The results presented here suggest that the tap withdrawal reflex is mediated by the five sensory neurons (ALMs, PLMs and AVM), and eight interneurons (AVAs, AVBs, AVDs, PVCs) that Chalfie et al. (1985) described, as well as three other neurons (PVDs and DVA). No role in mechanosensory integration for the LUA cells was demonstrated. Figure 7 shows the simplified anatomical connectivity of this circuitry. In general, the tap-withdrawal circuit can be roughly divided into circuitry designed to integrate anterior sensorimotor input and circuitry designed to integrate posterior sensorimotor input. These two subcircuits appear to functionally inhibit each other, and thus the behavioural output is the result of a balance of the activities of these two subcircuits. Of all the non-touch circuit neurons that were tested, only PVD and DVA could be implicated in the tap withdrawal reflex on the basis of the data presented here. However, it is possible that other cells may play small roles which may only be detected by ablating large numbers of neurons at the same time. Further ablation studies may detect these effects, but given that ablation of these cells on their own has no effect on the tap withdrawal response, it is unlikely that any of these cells will prove to play a major role in mechanosensory integration.

The effects of ablation of the two novel neurons (PVD and DVA) suggests a role for these cells in mechanosensory integration. Both PVD and DVA synapse with both the anterior

integration and posterior integration circuitry (see Table 1C; Figure 7). We suggest that a possible

functional role for PVD may be to provide a level of excitation to the circuitry. This excitation or

"tone" is either a reflection of background mechanosensory input, perhaps making the animal

more responsive in a noisy world, or it is a reflection of activity in the milieu interieur, in which

case PVD may be responding to paracrine or neurohumoral signals. PVD may play a sensory role

akin to that of a stretch receptor (Way & Chalfie, 1988 citing Ed Hedgecock) or a mechanism by

which dynamic gain control is achieved (Fischer & Carew, 1993). This possibility is supported

by the observations that PVD has virtually no partners presynaptic to it and has previously been

implicated in the integration of harsh touch (Way & Chalfie, 1989). The role played by DVA in

the integration of mechanosensory information is similar, however DVA is less likely than PVD

to be a mechanoreceptor. The connectivity of DVA is consistent with that of an interneuron

rather than that of a sensory neuron. It is a midline interneuron which receives input from

putative tail chemosensory neurons (the PHA cells are morphologically similar to other

chemosensory neurons, in that they have sensory endings in the phasmid sensilla through which

they can take up a fluorescein dye (Hedgecock, Culotti, Thomson & Perkins, 1985) and might

therefore modulate the tap response according to the chemical environment. Thus we have

assigned DVA a role as an interneuron in the tap withdrawal reflex circuitry.

An underlying assumption of this work is that the neuroanatomical connections described

by White et al. (1986) have some corresponding functionality. This assumption underlies the

main criteria used to identify candidate cells for ablation. Although it is unlikely that a cell with

sparse or absent anatomical links to those cells described by Chalfie et al. (1985) could have a

significant role in mediating the response to tap, it is possible that the functional links do not

necessarily correspond to the anatomical ones identified under an electron microscope. The

designation of a synapse was made by White et al. (1986) on the basis of the presence of a pre-

synaptic specialization visible in electron micrographs. All membranes adjacent to this specialization were designated as post-synaptic partners. Thus many of the anatomical synapses may not be functional (for example, some might lack the appropriate receptor phenotype). Also, this method of synapse identification is not sensitive to neurohumoral or paracrine effects. That is, any neuromodulator released into the neuropil might alter the circuit properties of the nematode nervous system and thus behaviour. This is especially a concern given the small size of the nematode. Multiple reports of circuit switching as a result of bath application or endogenous release of neuromodulators have been made (Getting, 1988; Getting, 1989; Harris-Warrick & Marder, 1991 for reviews) and an extensive battery of neuropeptides has been described in Ascaris, a related nematode (Stretton et al., 1992).

The results presented here support the relation between the anatomical connectivity and the functionality of synapses in the nematode. Specifically, because the tap withdrawal reflex can be quantified (unlike the response to touch, which is hand delivered) it has been possible to detect subtle effects of cellular ablation. Thus, the observation that both ALM- and AVM,ALM- animals invariably accelerate in response to tap, but the AVM,ALM- animals accelerate more vigorously suggests that the connections between AVM and the circuitry used in the posterior touch response are functional and inhibitory in nature as hypothesized by Chalfie et al. (1985). At the very least it can be said that AVM has an effect on mechanosensory integration that is independent of ALM, and thus provides more than just parallel processing of anterior mechanosensory information.

The relationship between circuitry and behaviour in C. elegans is robust and bi-directional. Changes in the nervous system as a consequence of ablation produce predictable changes in the form of the behaviour; observed changes in behaviour may provide information about the nature of the underlying nervous system. This latter approach may be used to assign

polarities (excitatory or inhibitory) to the chemical synapses studied in the tap withdrawal circuit. Hypotheses about what polarity configurations might best account for the behavioural observations reported here are difficult given the complexity of the circuitry involved. However, these hypotheses can be aided by the formulation of an appropriate computational model of the circuitry. Such a model could be used in conjunction with these studies to form specific predictions about the polarities of chemical synapses within the tap withdrawal circuit. The next experiment approaches the problem of defining these functional characteristics of the tap withdrawal circuit elements by using just such a model (Wicks, Roehrig & Rankin, 1996). This might in turn facilitate the determination of neurotransmitters and neurotransmitter-receptor pairings in these cells—work which is in its infancy.

Experiment 2: The determination of synaptic polarity

Although the detail of the anatomical data used to describe the tap withdrawal circuit is considerable, the functional polarities of the neurons of this circuit are unknown. This experiment describes an array of putative polarities for synapses of the tap withdrawal circuit. These predictions were made by optimizing the behaviour of a modeled circuit under conditions of degradation isomorphic to laser ablation of the circuitry underlying the tap withdrawal behaviour of real animals. Additionally, this model was used to investigate roles for two novel mechanosensory integration neurons. The model supports the conceptualization of PVD and DVA conveying mechanosensory and chemosensory information to the tap withdrawal circuit.

<u>Methods</u>

<u>The Model</u>.

Although the model was physiologically motivated, the absence of detailed physiological data from <u>C. elegans</u> made it necessary to make a number of extrapolations from the related nematode <u>Ascaris lumbricoides</u>. These assumptions are presented in physiological rather than mathematical form to ensure that they are realistic. Furthermore, preliminary investigations suggested that polarity predictions based on the modeled circuit were more strongly determined by circuit connectivity than the exact values of parameters used. Thus, rough ranges for these parameters rather than precise values were derived. The effects of varying some of the more uncertain parameters were then assessed by re-running the same experiments with values of these parameters varied over three orders of magnitude.

The circuitry was constructed by extracting connectivity data from AY's Neuroanatomy for Computation (Achacoso & Yamamoto, 1992). These data indicated not only the presence or absence of a set of synaptic contacts between a pair of neurons—referred to here as a synaptic

class—but also incorporated the actual number of documented electrical and chemical connections within that synaptic class. Each synaptic contact within a class of synapses was assigned the same reversal potential and conductance as all other synapses within that class. This enabled the simple construction of complex circuits in which all documented synapses (including all bilateral asymmetries) were included in the model. It was assumed that the functional efficacy of a synaptic class was correlated with the number of contacts observed within that synaptic class. Thus, circuits constructed in this way possessed connections with weights determined by anatomical criteria. These weights were not varied further in this model; only the reversal potential which determined the sign of the connection was allowed to vary. The complete connectivity of the modeled tap withdrawal circuit is shown in Figure 8.

The model was based on all available physiological and anatomical data from C. elegans and the related nematode, Ascaris. The physiological parameters used in the derivation of the data presented in this report are shown in Tables 2 and 3. The model was implemented in Objective-C on Intel-486, HP series 9000 and NeXT computers running NEXTSTEP software, and was integrated using fourth order Runge-Kutte (Press, Flannery, Teukolsky & Vetterling, 1989) to an accuracy of 0.5%. The mathematical formulation of the model, and its implementation are the contribution of Chris Roehrig, and have been relegated to Appendix 1 for reference purposes.

The Gearbox.

This model does not explicitly incorporate nematode locomotion. These issues have been dealt with adequately elsewhere (Erdös & Niebur, 1993; Niebur & Erdös, 1991; Niebur & Erdös, 1993). Rather, this report concentrates specifically on sensorimotor integration. However, since a behavioural variable was used to optimize the modeled output, it was necessary to rigorously define the relationship between the an animal's locomotion and activation of the circuitry which

Table 2: Average simulation parameters. The list of physiological parameters utilized in the four experiments run in this report are summarized. For a more detailed discussion of the origin of these values see Appendix 1.

NEURON PARAMETERS	VALUE	UNITS
Membrane Resistance	See Table 2	Ohms
Membrane Capacitance	See Table 2	Farads
Membrane Leakage Potential	-0.035	Volts
SYNAPTIC PARAMETERS		
EPSP Reversal Potential	0.00	Volts
IPSP Reversal Potential	-0.048	Volts
Synaptic Conductance	6.00E-10	Siemens
$V_{(Range)}$	0.035	Volts
Gap Junction Conductance	5.00E-09	Siemens
TAP PARAMETERS		
Pulse Rest	0	Amps
Phasic Pulse	1.00E-11	Amps
Start Time	0.01	Seconds
Duration	0.3	Seconds
Tonic Pulse	2.50E-10	Amps

Table 3: Summary of single neuron characteristics. Branching morphology and process length

were taken from Wood (1988b) and White et al., (1986) assuming a standard worm length of 1

mm. An average process diameter of 0.5 μm was obtained from measurements of electron

micrographs in White et al., (1986) and White et al., (1976). An average soma diameter of 5 μm

was measured from camera lucida drawings in Wood (1988b). $V(l)/V_o$ is the attenuation of a

voltage clamp V_o along the full length of the primary process according to a sealed end cable

equation (Rall, 1989) and gives an indication of a cell's isopotentiality.

Cell	Primary Process (mm)	Secondary Process (mm)	Surface Area (10^{-6} cm^2)	C_m (pF)	R_m (GΩ)	$\dfrac{V(l)}{V_o}$
ALM	0.50	0.03	9.1	9.1	16	0.89
PLM	0.48	0.06	9.1	9.1	16	0.9
AVM	0.24	0.03	5.0	5.0	30	0.97
PVD	0.74	0.22	16	16	9.4	0.78
PVC	0.96	-	16	16	9.4	0.68
AVA	0.93	-	15	15	10	0.69
AVB	0.86	-	14	14	11	0.73
AVD	0.86	-	14	14	11	0.73
DVA	0.91	-	15	15	10	0.70

Figure 8: The complete connectivity of the tap withdrawal circuit is shown. The circuit consists

of seven sensory neurons (shaded circles), nine interneurons (unshaded circles) and two

motorneuron pools (not shown) which produce forward and backward locomotion (triangles).

Chemical connections are indicated by arrows, with the number of synaptic contacts being

proportional to the width of the arrow. Gap junctions are indicated by dotted lines. Every

connection represented in this figure was also represented in the model. This representation is

useful for identifying connection asymmetries which might underlie the origin of oscillations

which control locomotion and are hidden in simpler views of the circuitry.
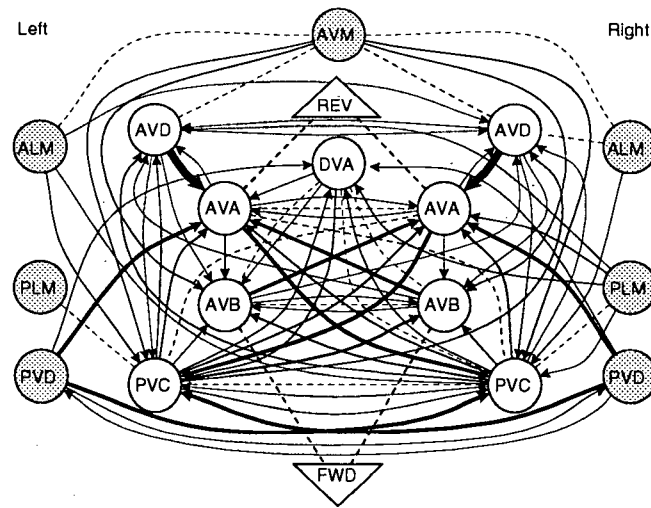
controls that behaviour. This issue was addressed with simple assumptions that were consistent

with work on the modeling of nematode locomotion (Erdös & Niebur, 1993; Niebur & Erdös,

1991; Niebur & Erdös, 1993; Stretton et al., 1992) and current theories of tap withdrawal circuit

function (Chalfie et al., 1985; Wicks & Rankin, 1995a). The output of the tap withdrawal circuit

was assumed to control locomotory behaviour primarily through the action of the two

interneurons AVB and AVA. These two interneurons make electrical connections with motor

neurons all along the ventral cord of the worm. The AVA interneurons make gap junctions with

the motor neurons (AS, VA and DA) that are presumed to excite backward locomotion; the AVB

interneurons form gap junctions with the motor neurons (VB and DB) which are presumed to

excite forward locomotion. Ablation of these cells almost completely destroys an animal's ability

to move forward (in the case of AVB ablations) or backward (in the case of AVA ablations)

(Chalfie et al., 1985; Wicks & Rankin, 1995a). Thus it was simply assumed that the degree to

which an animal reversed was proportional to the depolarization of the AVA interneuron and

inversely proportional to the depolarization of the AVB interneuron. Forward locomotion in

response to tap was also proportional to this value; a lower propensity to reverse was equivalent

to a higher propensity to accelerate. The exact nature of this proportionality was not defined

since in vivo it will be modulated by a number of neural, hydrostatic and physical forces that are

beyond the scope of this endeavor. The gearbox—that is, the transformation equation that was

used to convert depolarization of AVA and AVB into behaviour—was simply

$$\text{Propensity to Reverse} \propto \int V_{\text{AVB}} - V_{\text{AVA}} dt. \tag{2}$$

The integration was calculated from the time of the tap stimulation until either the end of the simulation or until the integrand changed sign. Additionally, the test for a change of integrand sign was suppressed for a grace period of 100 msec to allow for initial transients following the tap.

One consequence of the gearbox assumption is that, because of uncertainty regarding the exact nature of the proportionality between the output of the AVA and AVB interneurons and the magnitude of the evoked behaviour, comparisons of model data and empirical data must be limited to relative changes in response magnitude. Thus, such comparisons were made between data profiles that had been normalized about the mean of that polarity configuration's response level (see below). This measure detected changes in the levels of responding to tap produced by an ablation series, without being sensitive to the absolute response magnitude of a particular circuit configuration—information which in any case is meaningless in the context of the gearbox assumption.

<u>Strategy for Neuron Polarity Determination.</u>

The animal's response to a light mechanosensory stimulus has been intensively studied (Chalfie & Sulston, 1981; Chalfie et al., 1985; Wicks & Rankin, 1995a). Specifically, Chalfie and colleagues have described the circuitry that underlies the worm's reflexive response to a light touch to either its head or tail. This touch circuit was the starting point for the delineation of the circuitry responsible for the animal's response to a mechanically delivered stimulus (i.e., "tap") to the side of the substrate upon which the animal moves. An intact worm will generally respond to a tap stimulus with a cessation of forward motion, a reversal through some distance and a resumption of forward locomotion in a new direction. This response has been termed the tap withdrawal reflex (Rankin et al., 1990). The magnitude of the tap withdrawal reflex can be quantified by measuring the distance through which an animal reverses.

The circuitry underlying the tap withdrawal reflex (Wicks & Rankin, 1995a) has been identified using the laser ablation technique (Avery & Horvitz, 1987; Avery & Horvitz, 1989; Sulston & White, 1980). Ablations of neurons in this circuit can quantitatively and qualitatively alter an animal's response to tap. Three neuron classes, each composed of one or two cells, transduce the tap stimulus and segment the animal into two mechanosensory fields; anterior mechanosensory stimuli are transduced by the ALM and AVM cell classes and the posterior input is transduced by the PLM cell class. The AVA and AVB interneurons are required for normal locomotion, while the AVD and PVC interneurons couple anterior and posterior mechanosensory input respectively onto AVA and AVB.

The ablation strategy previously utilized to describe the tap withdrawal circuit resulted in the accumulation of datasets which represented the worm's response to a tap stimulus when individual neurons in the tap withdrawal circuit were destroyed with a laser microbeam (Wicks & Rankin, 1995a). For example, the ablation of either the ALM sensory neuron class alone, or in conjunction with the AVM sensory neuron resulted in animals which accelerated forward, rather than reversed, in response to a tap. Furthermore, the magnitude of both the reversal and acceleration behaviours elicited by a tap stimulus were modulated by ablation of the tap withdrawal circuit neurons. For example, the reversal response of animals lacking the PLM sensory neuron was larger than the reversal response of control animals with an intact circuit. This difference was presumed to be due to the loss of excitatory electrical input from the PLM sensory neuron to the PVC interneuron, as well as chemical input to the AVA and AVD interneurons (see Figure 8). This chemical input could be either excitatory or inhibitory, and that variable should, in theory, affect the magnitude of the reversal response observed. Similarly, the ablation of both the ALM and AVM sensory neurons resulted in larger accelerations in response to a tap than did the ablation of ALM alone.

The connectivity of the worm's nervous system has been well described. However, the signs or polarities of the synapses, excitatory or inhibitory, which determine the behaviour produced by a given circuit are unknown. This set of synaptic signs is referred to in this report as the polarity configuration of that circuit. The same connectivity may result in many distinct behavioural outcomes depending on the particular polarity configuration that the circuit possesses. The data that represents the animal's tap withdrawal behaviour following neuronal ablation—presented in Figure 9—reflects the polarity configuration of the worm's tap withdrawal circuit; it is a representation of how the circuit that is defined by that set of polarities responds, in various states of degradation, to a tap stimulus. Another circuit—one with identical connectivity but a different polarity configuration—would not necessarily yield the same profile of tap responses as a result of that series of lesions. Indeed, each possible polarity configuration of the tap withdrawal circuit might result in a distinctive pattern of behaviour in response to the ablation of individual, or small sets of, neurons. This profile of behaviour (see Figure 9), representing the real animal's response to a tap in a variety of ablation conditions, was used to find the best match within the space of all possible polarity configurations using a computational model of the tap withdrawal circuit.

We constructed a model of the tap withdrawal circuit and produced lesions in that circuit that were isomorphic to the ablation conditions represented in Figure 2. We then determined the profile of behaviour for all seven ablation conditions predicted by the model for an arbitrary polarity configuration. The fitness of that polarity configuration was determined by an error function with three terms (see Equation 3, below), each of which used a least squares error approach to compare the model data profile for that configuration and the ablation data profile (see Figure 10). Then, all possible polarity configurations were exhaustively enumerated and sorted according to their fitness. The response profiles (modeled and empirical) were compared

Figure 9: The mean response magnitude of the tap withdrawal reflex of seven groups of animals

are shown. This data was used to optimize the array of underlying functional polarities of the

modeled circuit. Some ablations (for example, the removal of PLM) resulted in larger reversal

responses than in control animals; other ablations resulted in consistent accelerations in response

to tap (indicated by a negative reversal magnitude for the ALM- and AVMALM- groups). Note

that the acceleration measure is a change in velocity whereas the reversal measure is a distance;

these are not directly comparable. Thus the ordinate represents a number which is proportional

to the amount of forward or backward locomotion. It was assumed that the pattern of responding

represented on this figure was partially dictated by the array of synaptic signs which constituted

the underlying circuitry (see Methods, Strategy for neuron polarity determination). These data

are adapted from Wicks and Rankin (1995a).
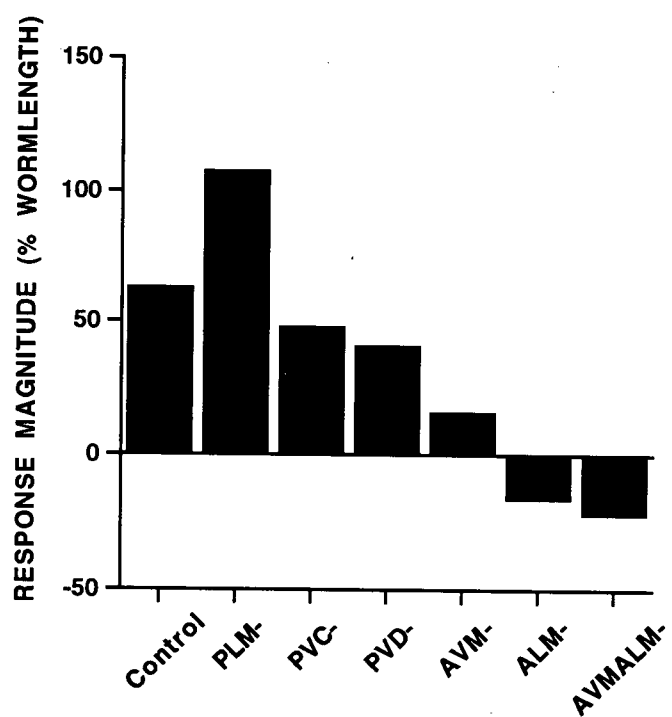
by first separating those conditions that were associated with reversal responses from those that were associated with accelerations. Then these two profiles were standardized by expressing each dataset as a series of Z-scores around the mean of each set of responses. This was done because we were interested in the relative change in the withdrawal response magnitude as a consequence of ablation, rather than the absolute value of the tap withdrawal response magnitude.

The first error term in the fitness function reflected how closely the ablation-induced modulation of modeled gearbox output matched the corresponding modulation of reversal behaviour in real animals. The reversal profile comprised those ablation conditions which resulted in reversals in the real animal: The intact, PLM-, PVC-, PVD- and AVM- conditions. Figure 10 shows three datasets which have been standardized to allow comparison. The empirical and modeled reversal profiles were each standardized as described above and compared. The reversal error term was simply the sum of the least-squared errors between the two reversal profiles. The second error term in the fitness function reflected how well the modulation of the acceleration behaviour of the model produced by ablation matched the corresponding modulation of acceleration behaviour in real animals. The acceleration profile thus comprised the two ablation conditions which resulted in animals that consistently accelerated in response to tap: The ALM- and AVMALM- ablations. Again, both the empirical acceleration profile (see Figure 10) and the modeled acceleration profile were standardized as described above and the least-squared error between the two acceleration profiles was derived for each configuration.

It was necessary to separate these two sets of comparisons because the acceleration and reversal behaviours were quantified with distinct measures (Wicks & Rankin, 1995a)—an acceleration is a change in velocity, whereas a reversal is a distance. Although these two responses were not quantitatively comparable, it was still possible to qualitatively evaluate the ALM- and AVMALM- ablation conditions with respect to the reversal profile in the model.

Any output from the gearbox (see Equation 2) associated with the acceleration profile should, in order to be considered in accord with data from the real animal, be on average lower than the output of the gearbox associated with the reversal profile. This statement reflects the fact that, regardless of the absolute value of Equation 2, in order to produce forward locomotion in response to stimulation the relative value Equation 2 must be more positive than the value required to produce a reversal. It is not possible to make any statement with respect to how much smaller this gearbox output should be because of the incommensurability of the acceleration and reversal measures, but accelerations should be associated with relatively lower gearbox outputs than reversals. Thus, a third error term was derived to evaluate whether the response type produced by the model was in accord with the empirical data in as much as the gearbox output for these conditions was on average lower than for those ablation conditions associated with the reversal profile. The mean gearbox value for the reversal conditions (Control, PLM-, PVD-, PVC- and AVM-) was calculated for both the empirical dataset in Figure 9 and the model dataset. A similar mean acceleration value was calculated for the acceleration conditions in the two datasets. These two sets of values were converted to Z-scores and a least-squares error between the model and empirical response type profiles was calculated. Since there were only two terms in each of these distributions, this comparison was strictly qualitative. If the gearbox output for the acceleration conditions was, on average, lower than the gearbox output for the reversal conditions, the two normalized distributions would be identical and the error contributed by this term would be zero. On the other hand, if the two acceleration conditions resulted in gearbox output that was, on average, larger than that produced by the reversal conditions the error contributed by this term would be positive and furthermore, would be insensitive to how

Figure 10: Two different response profiles from Experiment 2A—one representing a good fit and one representing a poor fit—are shown along with the empirical data to which they were compared. These datasets are expressed as three sets of standardized Z-scores used to evaluate the relative modulation by ablation of three behavioural measures: Reversal magnitude, acceleration magnitude, and response type. The first set of Z-scores incorporates those ablation configurations that result in a reversal response in the empirical dataset shown in Figure 9. It was used to determine the error associated with the relative modulation of reversal magnitude as a consequence of ablation between that dataset and each polarity configuration from the model. The second set of Z-scores similarly assessed the error associated with the relative modulation of acceleration magnitude as a consequence of ablation. A third set of Z-scores evaluated the qualitative fit between the model and the empirical profiles with respect to response type. It assessed whether the gearbox output (Equation 2) was lower, on average, for the acceleration profile than for the reversal profile, as was assumed to be the case for the intact animal. The fitness of a given configuration was calculated by summing the least-squared error between the model and empirical profiles for each of these three sets of standardized data. It is clear that the fitness of the modeled circuit to the behavioural data can be strongly modified by altering the array of underlying polarities in the modeled circuit.
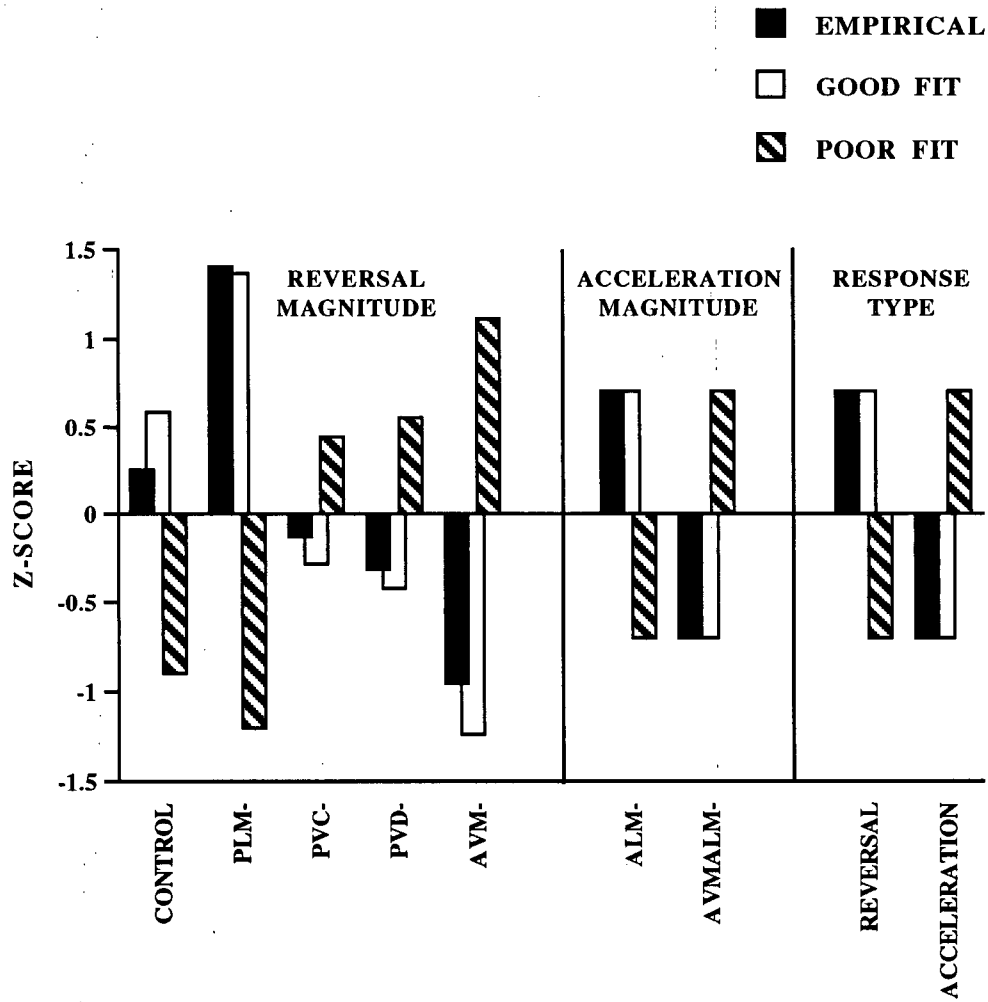
much larger the output was. Examples of two different response profiles from a typical experiment—one representing a good fit and one representing a poor fit—are shown in Figure 10 along with the empirical data to which they were compared.

These three error terms were summed for each configuration. Thus, the fitness measure which was used to sort the list of polarity configurations was given by,

$$Fitness = E_{\text{Reversal Magnitude}} + E_{\text{Acceleration Magnitude}} + E_{\text{Response Type}}, \tag{3}$$

where each term represented the least squared error associated with the comparison of the model and empirical profiles for that behavioural measure. After summing the three error terms associated with each polarity configuration, the entire list of polarity configurations was sorted according to the resultant fitness function. The top 50 sorted polarity configurations from a single experiment are shown in Figure 11. The interpretation of this list was complicated by the fact that it was both exhaustive and complete. The top n predictions, while being sorted according to absolute fitness, may not have been significantly different from each other statistically. To address this issue, the polarity of each cell was analyzed independently. A given neuron was characterized as excitatory or inhibitory if it was determined that a given polarity for that cell was clustered at or near the top of the list. For example, a cell that was predicted to be inhibitory for the top 50 configurations was more likely to actually be inhibitory than a cell whose predicted polarity alternated through the top 50 configurations, even if in the case of the best configuration, it was also inhibitory.

A sign test (Siegel, 1956) is a nonparametric statistic that can be used to assess the probability that the two possible polarities (+1 or -1 in this case) appear with equal frequency within a tested fraction of this sorted list. A significant sign test indicates that one or the other

Figure 11: Sample polarity configurations. The top 50 polarity configurations sorted according to error from Experiment 2A are shown. This circuit did not include the DVA interneuron and hence there were 256 possible configurations (2^6) in the complete sorted list. Thus, the top 10% of the list reported in Table 4A consists of the top 26 polarity configurations shown in this figure. The PVD sensory neuron class was not externally stimulated during this run. A polarity that is consistent with that which resulted from statistical considerations is shown as a lightly shaded box; a polarity that is not consistent is shown in an unshaded box. No polarity predictions were made for the AVA or DVA neurons. These columns are darkly shaded. In this experiment, the tenth and sixteenth configurations are entirely consistent with the consensus configuration predicted in this report.

	ALM	PLM	PVD	AVB	PVC	AVA	AVD	AVM	DVA		ALM	PLM	PVD	AVB	PVC	AVA	AVD	AVM	DVA
1	1	-1	-1	-1	1	1	1	-1		26	1	-1	1	-1	1	1	-1	-1	
2	-1	-1	-1	1	-1	-1	1	-1		27	-1	-1	-1	1	1	1	-1	-1	
3	-1	-1	-1	1	-1	1	1	-1		28	1	1	-1	-1	1	-1	-1	-1	
4	1	-1	-1	-1	1	-1	1	-1		29	-1	1	-1	1	1	-1	1	1	
5	1	-1	-1	-1	1	1	-1	-1		30	1	-1	1	-1	1	-1	1	1	
6	1	-1	-1	1	1	-1	1	-1		31	-1	-1	-1	-1	1	-1	-1	-1	
7	-1	-1	-1	1	1	-1	-1	-1		32	-1	1	-1	-1	1	-1	-1	-1	
8	-1	-1	-1	1	1	-1	1	-1		33	1	-1	1	1	1	1	-1	-1	
9	1	-1	1	1	1	1	1	-1		34	-1	-1	-1	-1	-1	1	1	-1	
10	-1	-1	-1	-1	1	-1	1	-1		35	1	-1	-1	1	1	-1	-1	-1	
11	-1	-1	-1	-1	1	1	-1	-1		36	1	-1	-1	-1	1	-1	-1	-1	
12	1	-1	1	-1	1	1	1	-1		37	-1	1	1	-1	1	-1	1	-1	
13	-1	-1	-1	1	1	1	1	-1		38	1	1	1	-1	1	-1	1	-1	
14	-1	-1	1	1	1	1	1	-1		39	-1	1	-1	-1	1	1	1	1	
15	-1	-1	1	1	1	-1	1	-1		40	-1	-1	1	1	-1	-1	1	-1	
16	-1	-1	-1	-1	1	1	1	-1		41	1	1	1	-1	1	1	-1	-1	
17	-1	-1	1	-1	1	1	1	-1		42	-1	1	1	-1	1	1	-1	-1	
18	1	-1	1	1	1	-1	1	-1		43	-1	-1	1	1	1	-1	-1	-1	
19	1	-1	-1	1	1	1	1	-1		44	-1	1	1	1	1	-1	1	-1	
20	-1	-1	1	-1	1	1	-1	-1		45	-1	1	1	-1	1	1	1	-1	
21	-1	-1	1	1	1	1	-1	-1		46	-1	1	-1	1	1	1	1	-1	
22	1	-1	-1	1	1	1	-1	-1		47	-1	-1	1	-1	1	-1	-1	-1	
23	-1	-1	-1	-1	-1	-1	1	-1		48	-1	1	1	1	1	1	1	-1	
24	-1	1	-1	-1	1	-1	1	1		49	1	1	1	1	1	-1	1	-1	
25	-1	-1	1	-1	1	-1	1	-1		50	-1	-1	1	-1	-1	1	1	-1	

sign clusters within that fraction of the sorted list at above chance levels. Thus, several one-sample sign tests were used for each neuron to determine if a given polarity for that cell clustered at a higher than chance frequency near the top of the sorted list of polarity configurations. This analysis was repeated for each of several fractions of the sorted list of configurations. As the sorted list of configurations was complete—that is, each configuration differed from all others—and since a significant prediction at one fraction of the list was indicative of a trend that might also be detected at other nearby fractions of the list if they were analyzed, this constitutes a conservative analysis of the data.

This experiment reports the results of analyses based on four fractions of the list of sorted polarity configurations. Three of these (the top 10%, first quartile, and top half) are arbitrary, but informative and the fourth (designated "Alpha") was based on the shape of the frequency distribution of the fitness function which was used to sort the list of polarity configurations. The Alpha fraction was defined as that fraction of the list of polarity configurations whose members possessed a fitness value that was more than one standard deviation above the mean of the fitness frequency distribution. Thus, the fraction of the list that was defined by Alpha was dependent upon the distribution of the error term used to sort the list. These fitness distributions are shown in Figure 12.

Modeling the Tap Stimulus.

The tap stimulus was modeled as a phasic depolarization of the sensory neurons (PLM, ALM, and AVM) which have been shown to mediate the response to tap in the intact animal (Wicks & Rankin, 1995a). However, it is likely that the tap does not represent the only input to the circuit in the intact animal. For example, the neuron PVD has been thought of as a stretch receptor (Way & Chalfie, 1988 citing Ed Hedgecock) or a background mechanosensory input detector (Wicks & Rankin, 1995a). In addition, the interneuron DVA receives considerable

synaptic input from chemosensory circuitry, and may therefore be a cell that modulates the activity of the tap withdrawal circuit according to the chemical milieu (Wicks & Rankin, 1995a). Therefore, four complete experiments were run to determine to what extent variations in the way these two cells were represented altered the nature of the polarity predictions. The effect of the inclusion of DVA was assessed by comparing two experiments in which DVA was included (Experiments 2 and 4) with two experiments in which DVA was absent (Experiments 1 and 3). Within each of these two conditions, the effect of the nature of the stimulation that PVD and DVA (when present) received was assessed. Two stimulation parameter sets were used. In the tonic condition (Experiments 3 and 4), the PVD and DVA neurons were activated by a low (one-quarter pulse input magnitude) tonic stimulation that was continuously present and not correlated with the phasic tap input to the other touch neurons. These parameters were chosen to mimic the effects of an unspecified mechanosensory and chemosensory input to PVD and DVA respectively. In the phasic condition, these two neuron classes were not explicitly stimulated (Experiments 1 and 2).

These results have been previously reported in published form (Wicks et al., 1996).

Results

The results of the four experiments are summarized in Table 4 (A-D). The table represents a series of probability values for sign tests conducted on the indicated percentages of the sorted lists of polarity configurations in each of four experiments: A) the exclusion of DVA with no stimulation of PVD, B) the inclusion of DVA in the circuit and no stimulation of DVA and PVD, C) the exclusion of DVA with tonic activation of PVD and D) the inclusion of DVA and tonic stimulation of DVA and PVD. Taken together, the results of the four experiments made significant predictions for the polarities of seven of the nine cell classes which constitute the tap withdrawal circuit. The strength of the prediction for a given neuron was roughly correlated with

the density of chemical connections that that neuron makes with other cells in the circuit (White et al., 1986). As it was the reversal potential of the chemical connections that was the critical factor in determining the fitness measure, no prediction would have been expected for neurons which make sparse chemical connections. All of the predictions from each of the four experiments were consistent. For example, if a cell was predicted to be inhibitory in the first experiment, any further predictions that were made in either the same or subsequent experiments were also inhibitory. As there were no disparities between the four experiments, a predicted polarity configuration based on consensus results was derived. This consensus configuration is shown in Figure 13. Because the polarity of two cells (DVA and AVA) were not reliably predicted at any level of significance, there are multiple configurations that are consistent with this consensus configuration.

For each experiment, the configurations were sorted according to their fitness. Figure 12 represents the frequency distribution of the fitness function from four experiments. Three points should be made regarding these distributions. First, each of these distributions was multimodal, suggesting that there were definable populations of configurations with good fitness. The frequency distributions were not evenly bisected as would have been expected if a single neuron polarity was critical to determining circuit function; a specific polarity for multiple neurons was required for good fitness. Second, the subpopulation of configurations with the best fitness was roughly delimited by the Alpha level (the fraction of configurations which were one standard deviation above the mean of the frequency distribution) in each of the four experiments. Finally, the mean of the two distributions that corresponded to experiments in which DVA and PVD were tonically activated (Figure 12 C and D) were both lower than the mean of those distributions which corresponded to experiments in which DVA and PVD were not explicitly

Table 4: The results of a sign test analysis of the frequency of occurrence of a given polarity in various sections of the sorted list of all possible configurations for four experiments (panels A-D) are shown. A significant p-value suggests that the indicated polarity for a given neuron occurs at a higher than chance level in the top $n\%$ of the list. The value "Alpha" represents that portion of the list which lies one standard deviation below the mean of the fitness distribution which was used as the sorting criteria, and is shown in Figure 12. ns indicates non-significance.

A	Cell	Polarity?	10%	Alpha(22.7%)	25%	50%
	ALM	Inhibitory	ns	$p = 0.0479$	ns	ns
	PLM	Inhibitory	$p < 0.0001$	$p = 0.0247$	ns	ns
	AVM	Inhibitory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	PVD	?	ns	ns	ns	ns
	AVB	?	ns	ns	ns	ns
	PVC	Excitatory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	AVA	?	ns	ns	ns	ns
	AVD	Excitatory	$p = 0.0290$	$p < 0.0119$	$p = 0.0169$	$p < 0.0001$

B	Cell	Polarity?	10%	Alpha(21.9%)	25%	50%
	ALM	Inhibitory	ns	ns	$p = 0.0421$	ns
	PLM	?	ns	ns	ns	ns
	AVM	Inhibitory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	PVD	Inhibitory	$p < 0.0001$	$p = 0.0153$	$p = 0.0011$	$p = 0.0208$
	AVB	?	ns	ns	ns	ns
	PVC	Excitatory	$p = 0.0002$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	AVA	?	ns	ns	ns	ns
	AVD	Excitatory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	DVA	?	ns	ns	ns	ns

C	Cell	Polarity?	10%	Alpha(19.9%)	25%	50%
	ALM	?	ns	ns	ns	ns
	PLM	Inhibitory	$p = 0.0094$	ns	ns	ns
	AVM	Inhibitory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	PVD	Inhibitory	ns	$p = 0.0007$	$p = 0.0016$	ns
	AVB	?	ns	ns	ns	ns
	PVC	Excitatory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0035$
	AVA	?	ns	ns	ns	ns
	AVD	Excitatory	ns	$p = 0.0003$	$p = 0.0002$	$p = 0.0011$

D	Cell	Polarity?	10%	Alpha(21.7%)	25%	50%
	ALM	Inhibitory	ns	$p = 0.0472$	$p = 0.0271$	ns
	PLM	Inhibitory	$p = 0.0018$	ns	ns	ns
	AVM	Inhibitory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	PVD	?	ns	ns	ns	ns
	AVB	Inhibitory	$p = 0.0110$	ns	ns	ns
	PVC	Excitatory	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0014$
	AVA	?	ns	ns	ns	ns
	AVD	Excitatory	$p = 0.0002$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	DVA	?	ns	ns	ns	ns

Figure 12: Fitness frequency distributions from four experiments are shown. The 256 possible

configurations in Experiments 2A and 2C and the 512 possible configurations from Experiments

2B and 2D were sorted according to the fitness measure described in the methods section. The y-

axis represents the number of configurations with a given error in each of 24 error bins. The

frequency distributions are multimodal. That fraction of the sorted list of configurations which

corresponds to the Alpha level (more that one standard deviation below the mean of the

distribution) lies to the left of the indicated Alpha level in each case. The mean of each

distribution is also indicated.
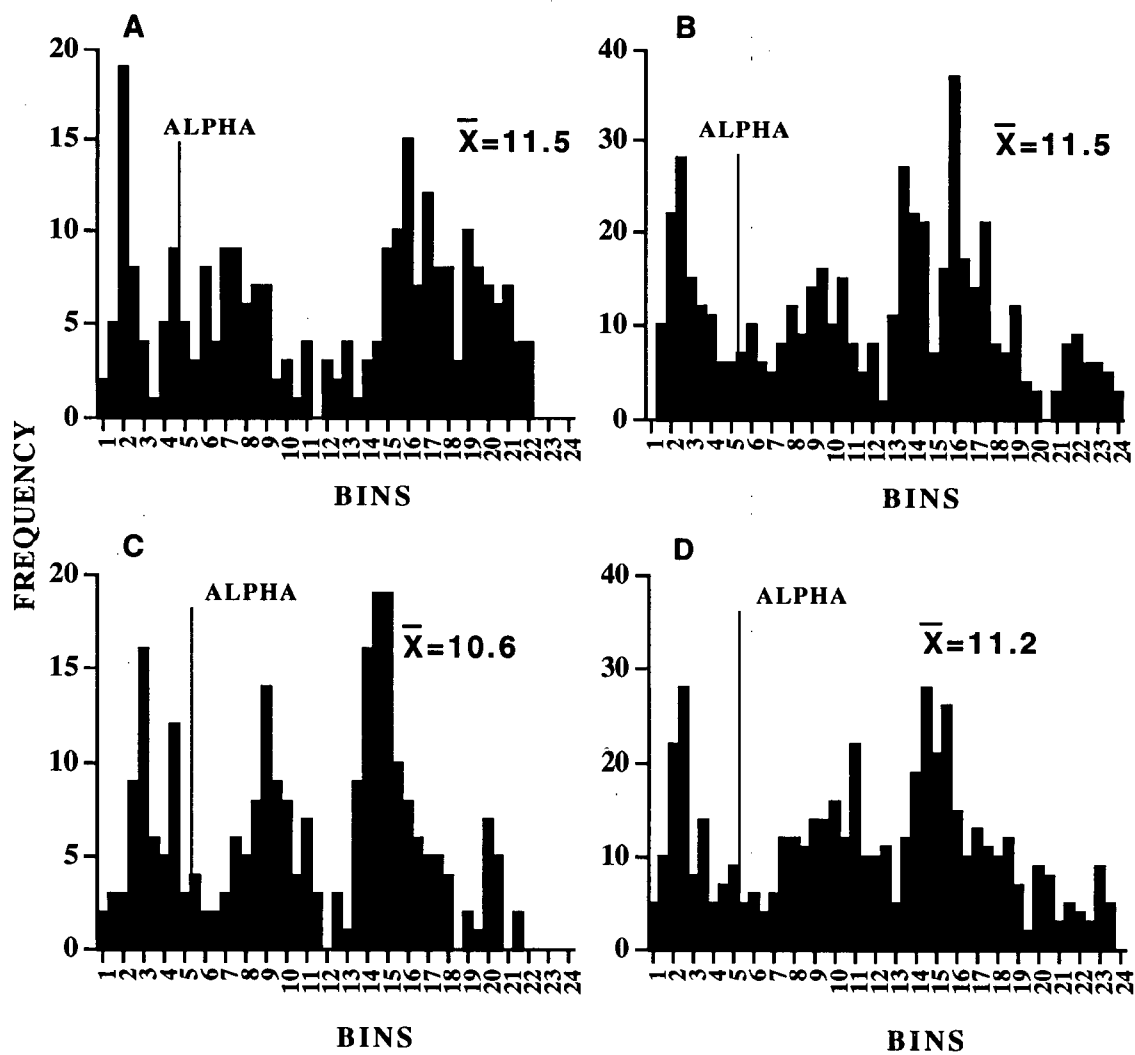
stimulated, suggesting that this manipulation increased the overall fitness. On the other hand, the inclusion of DVA (Figure 12 B and D) did not systematically improve the fitness, as measured by the fitness distribution means.

Experiment 2A.

In the first experiment, the circuit was stimulated with a phasic "tap" to the mechanosensory neurons (PLM, ALM and AVM), and was constructed without DVA. The best fifty polarity configurations from this run are presented in Figure 11. The results of this experiment are summarized in Table 4A. The three mechanosensory neurons (PLM ALM, and AVM) were predicted to be functionally inhibitory, although the prediction for ALM was fairly weak. For example, significantly more polarity configurations in the top 10% (the top 26 configurations) of the list shown in Figure 11 possessed inhibitory polarities for the AVM neuron than possessed excitatory polarities for that cell. In contrast, the polarities of the AVA, AVB and PVD neurons alternated with sufficiently high frequency in that same fraction of the list of configurations in Figure 11, that neither the excitatory nor inhibitory polarity could be said to predominate statistically for these cells. The two neurons (PVC and AVD) which couple the sensory input from the mechanosensory cells to the two neurons which control locomotion were both predicted to be excitatory.

Experiment 2B.

For the second experiment, DVA was added to the circuit and the same stimulation parameters were used. The results of this run are summarized in Table 4B. In this experiment, five of the nine cell classes possessed polarities which were correlated with good fit to behavioural data. The sensory neuron classes ALM and AVM were predicted to be inhibitory, although again, the ALM prediction was weak. Again, both of the neuron classes which modulate locomotion via mechanosensory input (PVC and AVD) were predicted to be excitatory (see

Figure 7). A prediction (inhibitory) was also made for the PVD cell class in this run. The DVA neuron class makes very sparse chemical connections with the rest of the circuitry, so no prediction for this cell was expected or obtained. In addition, no polarity predictions made for the AVB or AVA neuron classes.

The addition of tonic activation of PVD and DVA: The PVD cell class probably does not directly detect the tap stimulus; in the absence of AVM, ALM and PLM worms do not respond to tap even if PVD is left intact (Chalfie & Sulston, 1981; Wicks & Rankin, 1995a). Rather, its morphology and the behaviour of animals which lack this cell suggest that it may act as a stretch receptor (Way & Chalfie, 1988 citing Ed Hedgecock). It is also possible that the cell responds to some interoceptive cue which makes the animal more or less responsive in accord with the level of stimulation in its environment, analogous to the dynamic gain control system described by Fischer and Carew (1993). In either case, the cell may provide tonic input to the tap withdrawal circuitry rather than the phasic input provided by the tap. Similarly, the DVA interneuron which receives heavy input from cells which carry chemosensory information may modulate the animal's responsiveness according to the nature of the chemical environment (Dusenbury, 1974). This kind of input, like the proposed PVD input described above, was modeled by applying a low tonic stimulation of the DVA interneuron in situ. Thus, two additional experiments were run which were identical to the first two except that the PVD and DVA cell classes received low tonic depolarization throughout the duration of the simulations.

Experiment 2C.

In the first of these two experiments, the circuit was constructed without DVA, and PVD was tonically activated at one-quarter the intensity of the phasic tap stimulus. The results of this run are shown in Table 4C. This experiment can be directly compared to Experiment 1 to assess the impact of tonic stimulation. The main difference between these two results is that when the

PVD cell class was activated, its polarity became more highly correlated with a good fit to the behavioural results with a concurrent decrease in the correlation of an inhibitory polarity for the mechanosensory neurons PLM and ALM. The predictions concerning polarities for PVC, AVD and AVM were maintained from Experiment 1-3.

Experiment 2D.

In the last experiment, the modeled circuit was stimulated by a phasic input to the mechanosensory neurons (PLM, ALM, AVM) and a tonic input to both the DVA and the PVD neurons, and all possible polarity configurations were assessed. The results of each of the one-sample sign tests used to assess potential clustering of a specific polarity are shown in Table 4D. The three mechanosensory neurons which were stimulated in this experiment (PLM, ALM, AVM) were predicted to be inhibitory. Of the six other cell classes tested in this run, a prediction was made for the polarities of three of them. Of the two neurons which constitute the gearbox (AVA and AVB), AVB was predicted to be inhibitory. The two neurons (AVD and PVC) which connect much of the mechanosensory input to the driver neurons were again predicted to be excitatory. No prediction was made for either the DVA neuron or the PVD neuron. It is somewhat surprising that no prediction was made for PVD in this run. Given that Experiment 2C suggested that tonic activation of PVD increased the correlation between an inhibitory polarity for that cell and a good fit to the behavioural data it was expected that this relationship would be maintained in experiment four since in this experiment, PVD was also tonically stimulated. However, there appears to be an interaction between the way PVD is stimulated and the presence of DVA.

These results can be contrasted with those from the third experiment (2C) to again assess the impact of the addition of DVA to the mechanosensory integration circuitry. As in the first two experiments (2A and 2B), the addition of DVA to the circuitry had several effects. First, it

Figure 13: Simplified circuit with predicted polarities. The circuit which mediates the nematode tap withdrawal reflex consists of seven sensory neurons (squares), nine interneurons (circles) and two motorneuron pools (not shown) which produce forward and backward locomotion (triangles). All cells represent bilateral classes of cells except AVM and DVA which are single cells. Chemical connections are indicated by arrows, with the number of synaptic contacts being proportional to the width of the arrow. Gap junctions are indicated by dotted lines. This circuit has been simplified for ease of presentation in two ways: First, the bilateral symmetry of the circuit has been collapsed; second, only classes of connections with an average of greater than five synaptic contacts are shown. The consensus polarities of the neurons in this circuit which were derived from four experiments are also shown. Predicted excitatory connections are green, whereas predicted inhibitory connections are red. Two neurons (AVA and DVA) did not posses polarities which clustered at above chance levels in any of the experiments presented in this report.
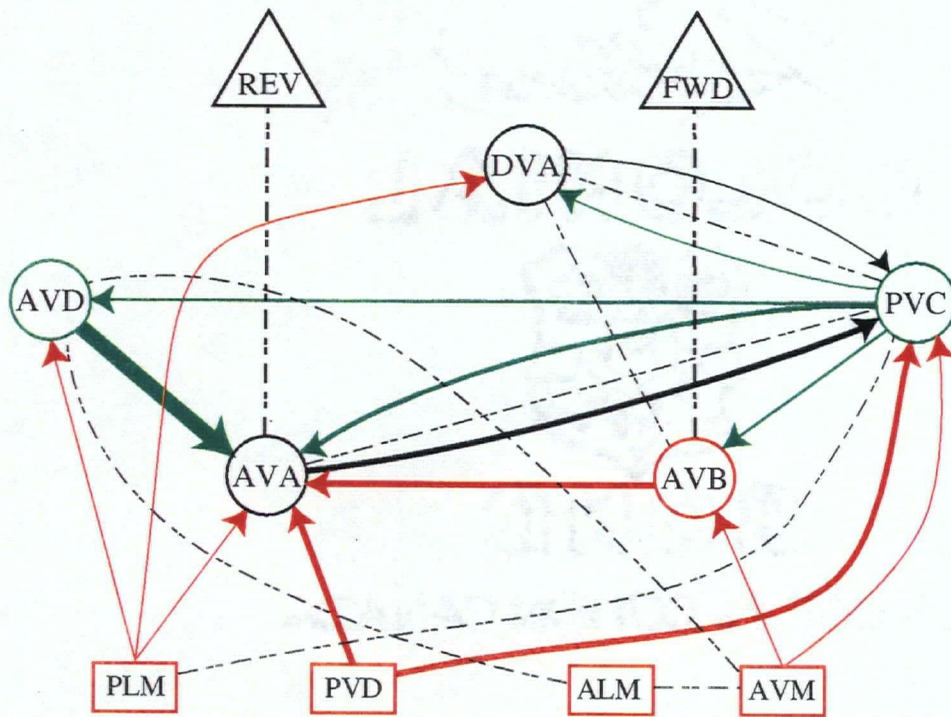
increased the correlation between the polarities of two cell classes and a good fit to behavioural data; ALM and AVB did not possess significant polarities in experiment three, but did in Experiment 2D. A determined polarity for one cell class (PVD) became less correlated with a good fit to behavioural data. Again, this result is surprising, because in the first two experiments (2A and 2B), PVD was predicted to be inhibitory only when DVA was present.

The results of Experiment 2D were compared to those from the Experiment 2B to allow an assessment of the effect of tonic stimulation with DVA in the circuit. There was a decrease in the significance of the polarity of PVD relative to run two as a consequence of tonic activation of that neuron. However, the main difference between these two runs was that AVB was predicted to be inhibitory in the last run but not the second. Thus, the addition of tonic stimulation of PVD and DVA increases the relative significance of the polarity of AVB.

Overall, the strongest set of predictions were made for the AVM, PVC and AVD cell classes. The AVD class makes among the most dense set of chemical synaptic connections within the animal (Achacoso & Yamamoto, 1992 , see Figure 8; White et al., 1986), so its predicted polarity would be expected to be important in the determination of tap withdrawal circuit behaviour. Similarly, the AVM class makes most of the chemical connections from the anterior mechanosensory field of the animal (White et al., 1986) and thus its polarity would also be expected to be critical in shaping an animal's response to a tap stimulus.

Finally, since polarity configurations which were consistent with the consensus configuration appeared several times in each experiment (due to redundancy of the polarities of neurons which did not achieve significance; see Figure 11), a mean consensus profile was constructed for each of the experiments. These mean consensus profiles (see Figure 14) produced remarkably similar fits to the target profile. Regardless of the stimulation parameters tested, or whether DVA was present in the circuit, these results all differed from the target profile in two

Figure 14: Mean consensus configuration fit. The best-fit profiles from four experiments are show in comparison to the empirical data to which they were compared for those configurations which were consistent with the consensus configuration in Figure 13. These datasets are each expressed as Z-scores around the mean of that dataset because only changes relative to the intact condition are interpretable. All four simulated datasets differ from the intact condition in two consistent ways. First, the PVD ablation had a large effect on the reversal magnitude of real animals, but had little effect on the modeled response. Second, the relative effects of touch-cell ablations in the model are not consistent with the changes produced by ablations in worms. Specifically, the relative effects of the AVM and PLM ablations are reversed in the model as compared with the worm. This may be due to mechanical processes which affect the transduction efficiencies of these touch cells. Error bars indicate SEM.
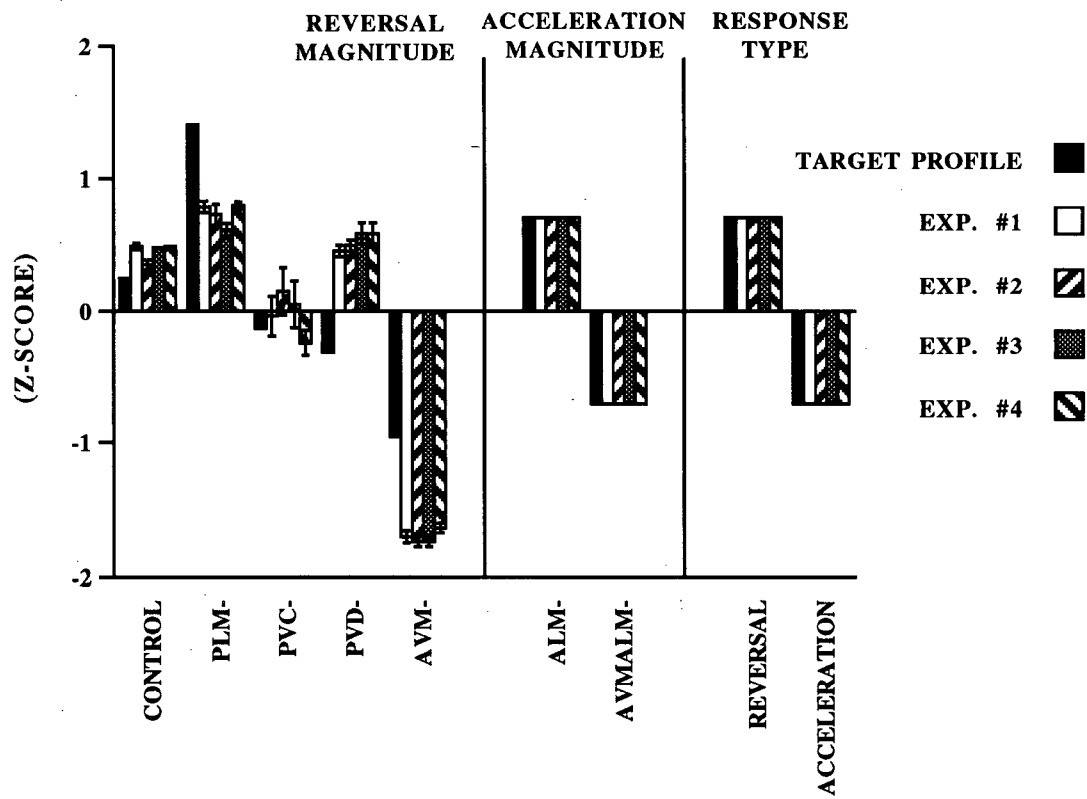
consistent ways. First, under all four experimental conditions, the relative effects of ablation of the sensory neurons on the behaviour of the model did not correspond to that observed in vivo . That is, the ablation of AVM in the model produced a larger relative change in behaviour than was observed in the worm whereas the ablation of PLM in the model produced a smaller relative change in behaviour than was observed in the worm. It was an assumption in these experiments that the transduction efficiency of all of the sensory neurons was the same, however, it is likely that this is not the case. The sensory processes of the PLM neurons are situated on the lateral aspects of the worm, whereas the AVM process runs in the ventral aspect of the worm (Chalfie & Sulston, 1981) and therefore may be differentially activated by a tap stimulus. Second, the ablation of PVD in the animal resulted in a significant decrease in the magnitude of the withdrawal reflex. In all of the best fit configurations, including the consensus configuration, the ablation of PVD had little effect on the output of the modeled circuit.

The effect of varying synaptic conductance.

Two physiological parameters (the gap junction conductance, $\hat{g}$, and the maximal synaptic conductance, $\bar{g}$, were each varied over two orders of magnitude to confirm that the exact values of these parameters were not critical in the prediction of neuron polarities. Experiment 2A was replicated under four distinct parameter conditions. In Experiments 2E and 2G the gap-junction conductance was increased ($\hat{g}$ = 5 × 10^{-8} S) and decreased ($\hat{g}$ = 5 × 10^{-10} S) respectively from the value used in Experiment 2A. In Experiments 2F and 2H the synaptic conductance was increased ($\bar{g}$ = 6 × 10^{-9} S) and decreased ($\bar{g}$ = 6 × 10^{-11} S) respectively from the value used in Experiment 2A. The results of these experiments are presented in Table 5. Although the number of predictions made in these further experiments was variable, there were no contradictions between these results and those reported earlier. In general, any manipulation which decreased the ratio of $\hat{g}$ to $\bar{g}$ decreased the number of predictions made by the model.

Although these manipulations certainly did have effects both on the absolute values of the propensity to reverse of any given polarity configuration and on the exact order of polarity configurations, the strategy used to predict neuron polarities appeared to be relatively insensitive to the exact parameters used in the simulation. This is consistent with preliminary versions of these experiments in which neurons were modeled as electrical circuits and synapses as entirely linear entities. Under these conditions—in which parameters were not even physiologically motivated, let alone justified—the strategy used to form predictions concerning synaptic character yielded the same set of predictions as are presented here.

Table 5: The effects of varying the values of the maximal synaptic conductance ($\bar{g}$) and the gap conductance ($\hat{g}$) over three orders of magnitude on polarity predictions are shown. Experiment 2A was replicated with a the gap junction conductance increased (Experiment 2E) or decreased (Experiment 2G) an order of magnitude or with the synaptic conductance increased (Experiment 2F) or decreased (Experiment 2H) an order of magnitude. Although the number of predictions varied, no changes in the consensus configuration were suggested as a consequence of these manipulations.

Cell	Exp. 2A	Exp. 2E	Exp. 2F	Exp. 2G	Exp. 2H	Consensus
ALM	Inhibitory	Inhibitory	?	?	?	Inhibitory
PLM	Inhibitory	?	?	?	?	Inhibitory
AVM	Inhibitory	Inhibitory	Inhibitory	Inhibitory	Inhibitory	Inhibitory
PVD	?	?	?	?	Inhibitory	Inhibitory
AVB	?	Inhibitory	?	?	?	Inhibitory
PVC	Excitatory	Excitatory	Excitatory	Excitatory	Excitatory	Excitatory
AVA	?	?	?	?	?	?
AVD	Excitatory	Excitatory	?	?	Excitatory	Excitatory
DVA	?	?	?	?	?	?

Discussion of Experiments 2A - 2H

The use of a simple computational model of the nematode tap withdrawal circuit has allowed the prediction of an array of synaptic polarities of the neurons that compose the circuit. The electrophysiological intractability of the worm's nervous system has previously made it very difficult to address this problem. However, the availability of datasets describing the behaviour of animals with lesions to the tap withdrawal reflex circuitry (Wicks & Rankin, 1995a) suggested a novel approach. A model of the circuit was produced and lesions in the modeled circuit that were isomorphic to those produced in vivo were made. The behaviour of real animals was then used as a target to which the output of the modeled circuit was optimized by altering the array of underlying polarities within the model. The approach outlined in this experiment has not been utilized before. In general, traditional neural network simulations, although motivated by behavioral or physiological observations, are not as tightly constrained by anatomical observations as is this simulation. It is more common to start with a fully interconnected multiple-layered network of nodes with weights defined arbitrarily and use a training method (like back-propagation) to converge on a solution that satisfies some performance criterion by varying the connection weights. In addition, the evaluation of the output of neural networks is generally treated directly. In this report, a statistical approach was utilized to evaluate network output across a range of experimental conditions.

In addition to predicting polarities for seven of the nine cells in the circuit, experiments were run to assess the impact of two other variables: 1) the tonic stimulation of DVA and PVD and 2) the removal of DVA from the mechanosensory integration circuitry. These tests provide some support for the tonic stimulation of PVD and DVA by background mechanosensory and chemosensory cues; less discrepancy between the model and behaviour of worms was observed in runs which included low tonic stimulation of these cells. In addition, a prediction for the

polarity of AVB was made only in a run which included tonic stimulation. The removal of DVA neither systematically improved the model's fit to behavioural data nor resulted in more polarity predictions for tap withdrawal circuit neurons.

<u>Sensory neurons.</u>

The first generalization which can be drawn from the results of these experiments is that neurons naturally cluster according to the array of predicted polarities into groups which correspond to anatomically or genetically defined classes. The sensory neurons modeled in this report fall into two broad classes: The touch cells (PLM, AVM and ALM; Chalfie & Sulston, 1981) and the putative stretch receptor PVD. The touch cells are a genetically distinct group of neurons. They express an array of active promoters which result in the production of a distinct neuronal morphology which is crucial to the mechanosensory function of these neurons (Chalfie & Sulston, 1981; Savage et al., 1989; Way & Chalfie, 1988). Given that these cells share a common genetic program, it might also be expected that they share a common neurotransmitter phenotype. The results presented here are consistent with this speculation; all three of these cells were predicted to be inhibitory.

It has been hypothesized that the role of the chemical connections from these sensory neurons onto the interneurons is to functionally inhibit the inappropriate response (Chalfie et al., 1985; Rankin, 1991; Wicks & Rankin, 1991; Wicks & Rankin, 1995a). For example, if the PLM sensory neurons were strongly activated then it would promote forward locomotion by exciting PVC (and consequently AVB; see Figure 13) via coupling to that cell, as well as simultaneously reduce backward locomotion via the inhibition of AVD and AVA. Behaviourally, a key distinction between the tap response and the touch response is that the tap stimulus activates both the anterior and posterior touch cells concurrently, making the behavioural output of the circuit a balance between the relative levels of activation within two subcircuits (Wicks & Rankin,

1991; Wicks & Rankin, 1995a). The functional inhibition of antagonistic subcomponents of behaviour is a critical and potentially informative aspect of the tap withdrawal circuit function.

Interneurons.

The two interneurons (AVD and PVC) which were predicted to be excitatory also fall into an intuitive functional category first proposed by Chalfie et al. (1985). These neurons each couple electrical input from the appropriate sensory neuron field onto the interneurons which drive locomotion. Thus AVD receives electrical input from the animal's anterior mechanosensory field and relays that information onto the AVA interneuron to drive backward locomotion. The polarity of the connection between AVD and AVA was predicted to be excitatory, which is consistent with this hypothesized role. Similarly, the connection between PVC and AVB was also predicted to be excitatory.

Of the two interneurons which drive the motor neurons responsible for locomotion (AVA and AVB) one was predicted to be inhibitory. This predicted polarity might appear to be inconsistent with the role that these cells appear to play in circuit function—the excitation of locomotion. However, since gap junctions from these neurons onto motor neurons in the ventral cord presumably mediate that excitation, the predicted inhibitory phenotype of these neurons would not contradict that role. Again these two cells each appear to perform the same function for the two subcircuits of the tap withdrawal reflex—one mediating forward locomotion and one mediating backward locomotion—and hence form a functional class. Although in these experiments, no prediction for the polarity of AVA was made, we would predict, based on a conservation of class function, that AVA is more likely to be inhibitory than excitatory.

The PVD sensory neuron was predicted to be inhibitory whether it was tonically activated during the simulation or not (although no prediction was made for PVD if it was tonically activated and DVA was included in the circuit; see Table 4). The cell configuration of

PVD and its two postsynaptic partners (PVC and AVA) may act as a dynamic gain control for the tap withdrawal circuit in the same way as described for the L30-L29 interneuron pair (and the synaptic input to these neurons from the siphon sensory neurons) in the Aplysia siphon withdrawal reflex (Blazis, Fischer & Carew, 1993; Fischer & Carew, 1993). In essence, these cells may be sensitive to the amount of mechanosensory stimulation that the animal experiences and modulate the animal's response accordingly. In C. elegans PVD may provide the input to this functional subcircuit. If AVA may be considered inhibitory, then this motif—an inhibitory and an excitatory cell each connected by both electrical and chemical connections and each receiving input from the same sensory source—may represent a general biological solution for gain control by a small neural subcircuit.

The polarity of the interneuron DVA was not explicitly predicted by this model presumably because this neuron makes few chemical connections within the circuit. However, the cell does make significant electrical connections with both PVC and AVB, and therefore may play a role in the integration of a tap stimulus. Although the largest number of polarity predictions was made in Experiment 2D when DVA was present, the best fit to behavioural data (Experiment 2C) as gauged by the mean of the fitness distribution was observed when DVA was removed from the circuit. Thus we are unable to confirm a role for DVA on the basis of these results. A more thorough treatment of the possible chemosensory role of DVA in the modulation of locomotion might explicitly incorporate the chemosensory input (Bargmann & Horvitz, 1991) and more formally model chemosensory gradients, perhaps incorporating the methods of Lockery et al. (1993).

Although a single neurotransmitter may have both an excitatory and an inhibitory effect postsynaptically, depending on what receptor that postsynaptic partner expresses at that synapse, these experiments assume that this is a rare occurrence. There is, however, evidence in

C. elegans of at least one example of a single neurotransmitter having both an excitatory and an inhibitory effect. The neurotransmitter GABA has been shown to have both functionally excitatory and inhibitory effects in the worm (McIntire, Jorgensen, Kaplan & Horvitz, 1993). Specifically, the use of laser ablation studies combined with the study of a mutant that eliminates the expression of GABA (*unc-25*) suggests that GABA release from AVL and DVB act to excite the enteric muscles of C. elegans. To investigate the possibility that a single tap withdrawal circuit neuron has dual synaptic character, the polarity of all synaptic classes from a single neuron would have to be allowed to vary independent of all others. Furthermore, each synaptic class could be allowed to assume one of multiple reversal potentials, or specifically increase or decrease postsynaptic conductance (Getting, 1989). These changes might disambiguate the function of synaptic classes which remain unclear even in light of these results. Chalfie et al. (1985) points out that it is difficult to intuit a role for a number of touch circuit synapses (for example the PVC to AVD class) and suggests that such classes may prove critical in determining the timing of sensorimotor integration. This hypothesis could bear further examination using the method presented here. These changes, along with a more complete investigation of the effects of varying the physiological parameters in this model could be implemented by adopting a more relaxed mechanism to search a larger parameter space, such as a genetic algorithm.

Finally, this experiment makes a rather significant assumption regarding synaptic efficacy: Functional weight is proportional to anatomical density. It should be noted that this assumption would probably not hold up to scrutiny. Specifically, an emerging principle regarding small biological networks is that functional the weight of a synaptic class is highly modulated by recent physiological, behavioural and neuromodulatory events (Getting, 1989; Harris-Warrick & Marder, 1991; Selverston, 1988). Both the prevalence of neuropeptides in Ascaris (Stretton et

al., 1992) and the conservatism inherent in the evolution of nervous system functioning suggest that the nematode nervous system is no exception to this general rule.
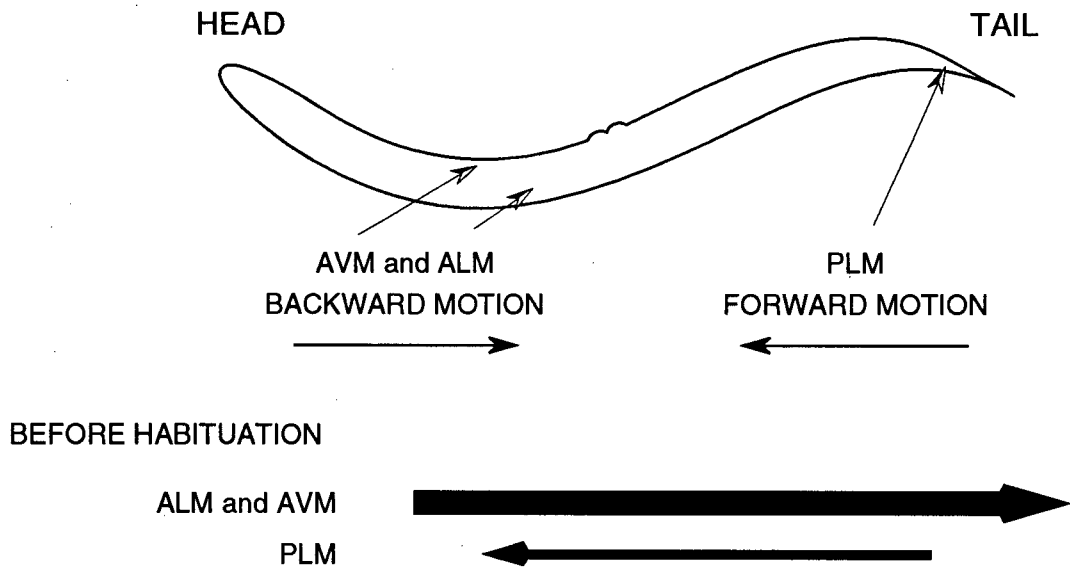
Many neurotransmitter types have been identified in C. elegans including GABA (McIntire et al., 1993), dopamine (Sulston, Dew & Brenner, 1975), serotonin (Horvitz, Chalfie, Trent, Sulston & Evans, 1982; Loer & Kenyon, 1994), octopamine (Horvitz et al., 1982), glutamate (Arena, Liu, Paress, Schaeffer & Cully, 1992) and acetylcholine (Chalfie & White, 1988). However, the neurotransmitter receptor pairings of the nematode tap withdrawal circuit neurons are as yet unknown. The predictions made in this report should facilitate the identification of putative neurotransmitters in this circuit. Furthermore, the method used to make these predictions may be generalized to determine properties of other neural circuits that mediate behaviours modulated by lesion.

Experiment 3: Habituation Kinetics of Antagonistic Reflexes

The first two experiments delineated the circuitry that mediated a reflexive response and suggested that the intact response is a summation of two underlying components. One characteristic of reflexive behaviour is that it often undergoes adaptive modulation as a consequence of experience. Habituation—perhaps the simplest form of learning—is expressed as a decrement in a reflexive behaviour (Groves & Thompson, 1970; Ratner, 1970; Sokolov, 1963; Thompson & Spencer, 1966). Certainly few, if any, reflexes exist which are entirely independent of the remainder of an animal's behavioural repertoire. Rather, a central tenet of neuroethology is that the production of a given response is dependent both on extrinsic cues, such as releasing stimuli, and on intrinsic cues, such as the state of any antagonistic reflex system (Fentress, 1983). Thus, during habituation of a response which is known to be determined by multiple underlying components, it is reasonable to ask whether changes in the intact response are due to changes in a single component or in multiple components of that response. This concept has been addressed theoretically by Ratner (1970), who presented a theory of habituation based largely upon response competition, and by Groves and Thompson (1970), who suggested that habituation of a given behaviour is a product of changes in antagonistic decrementing and facilitating processes.

The nature of an animal's response to tap—that is, acceleration or reversal—is determined by the relative input from at least two distinct mechanosensory fields (see Figure 15): Anterior input is transduced by three neurons (the bilateral ALM neurons and the midline AVM neuron), and posterior input is transduced by a single pair of neurons, the PLM sensory neurons (Chalfie & Sulston, 1981; Chalfie et al., 1985; Wicks & Rankin, 1995a). The behavioural output in an intact animal is an integration of these two competing responses. The withdrawal reflex can be modulated in predictable ways by ablating neurons within the circuit (Wicks & Rankin, 1995a).

Figure 15: Reflexive responding mediated by antagonistic inputs. The worm's response to a tap stimulus is determined by the relative levels of two antagonistic mechanosensory inputs.

HEAD   TAIL

AVM and ALM   PLM
BACKWARD MOTION   FORWARD MOTION

BEFORE HABITUATION

ALM and AVM

PLM

This experiment was designed to examine whether changes in behavioural output in intact animals that occur during habituation can be reconciled with habituation of both the forward and backward tap withdrawal response revealed by laser ablation of selected sensory inputs.

Materials and Methods

Subjects.

A total of 188 hermaphroditic Caenorhabditis elegans of the wild-type (N2) strain were used. First stage larval (L1) animals were obtained 2-3 hr post-hatching as previously described (Wicks & Rankin, 1995a) and prepared for single-cell laser microsurgery. Data on the response to the first stimulus in the habituation series obtained from some of these animals were reported in detail in Experiment 1.

Procedure.

Single-cell laser ablations were performed as described previously (Wicks & Rankin, 1995a). Ablations were performed by targeting identified neurons and neuron precursors (Sulston & Horvitz, 1977) in five groups of animals. Two groups of worms (PLM- and PVC-) underwent bilateral removal of neurons which resulted in animals which consistently reversed to tap. These ablations disrupted the posterior mechanosensory circuitry: PLM- animals lacked the posterior microtubule neurons, while PVC- animals lacked the gap junctioned input from the tail-touch receptors which is required for posterior-touch induced forward locomotion. The PLM- animals respond to tap with larger reversals than controls, whereas PVC- animals respond to tap with reversals of the same magnitude as controls (Wicks & Rankin, 1995a). A third group of animals (ALM-) underwent bilateral removal of the anterior lateral microtubule neurons. Previously, this treatment been shown to result in consistent accelerations in response to tap (Wicks & Rankin, 1995a). A fourth group of animals (AVMALM-) underwent a surgical removal of the anterior ventral microtubule neuron (AVM) in addition to the removal of the ALM neurons. The AVM

neuron was removed by ablating the Q_R blast cell from which AVM is derived. This procedure results in larger accelerations than are observed in ALM- animals alone (Wicks & Rankin, 1995a). Sham surgical controls were anaesthetized and recovered as were the other animals but no ablations were performed. Animals from each ablation group were partitioned further into groups to be run at either a 10 s interstimulus interval (ISI) or a 60 s ISI. Individual animals were raised alone on food-seeded plates and underwent habituation training between 3 and 4 d post hatching.

Habituation training was conducted as described in Rankin et al. (1990) with the following modifications. Since animals were raised on individual plates seeded with E. coli in this study, and since it was a high priority that animals not receive any mechanosensory stimulation prior to habituation training, animals were tested on the same plates as they were raised on. As a consequence, animals were tested in the presence of a bacterial lawn (E. coli). Individual plates were placed gently under the optics of the dissecting microscope and allowed to rest for 1 to 2 min. Then 40 tap stimuli were delivered to the plates at either 10 or 60 s intervals and the behaviour was videotaped. Animals were dishabituated with a mild shock delivered to the agar spanning the worm at the next scheduled tap as previously described (Gannon & Rankin, 1995). The worms were allowed to recover from the nonspecific effects of the shock for between 20 s and 30 s and then dishabituation was assessed with a series of three taps delivered at the same ISI as the training taps. Although this brief recovery period was deemed necessary to ensure that the worms were prepared to respond to the next tap, it is possible that this period was also sufficient to allow some recovery from habituation. Previously (Rankin & Broster, 1992), it has been demonstrated that the tap withdrawal reflex of worms habituated at a 10 s ISI showed no significant recovery 30 s after training, but is largely recovered after 5 min. The tap withdrawal reflex of animals trained at a 60 s ISI showed no significant recovery even after 5 min. This

suggests that any elevation in responding detected after the shock in these experiments should have been due to the shock itself, rather than to the brief time allowed to recover from the shock.

Analysis.

Most statistical comparisons were made using an ANOVA with Fisher's PLSD post-hoc tests (Statview, Abacus Concepts, 1992) where appropriate. A repeated-measures ANOVA was used to analyze the incidence of accelerations during habituation in control groups which emitted both responses. For each animal, the total number of accelerations in each block of ten stimuli during training was calculated and analyzed across ISI.

As previous results clearly showed a characteristic decrement of the magnitude of the tap withdrawal reflex as a consequence of repeated stimulation (Rankin et al. 1990; Rankin and Broster, 1992; Broster and Rankin, 1994) a one-tailed t-test for each group comparing the initial response magnitude to the mean magnitude of the last three responses in the habituation series, was used to assess habituation. Two dependent measures were used to characterize the shape of a habituation curve: Initial rate and asymptotic level. Since it was likely that the initial rate of habituation was related to initial response magnitude, and since we were concerned particularly with comparing rates of habituation between groups with significantly different initial levels of responding (Wicks and Rankin, 1995), we standardized all data such that the mean of the initial response magnitude of each group was equal to 100 %. Thus all habituation was expressed in terms of percent decrement from initial levels.

As no parameter constraints were used in the derivation of best-fit curves, the curve fitting procedure occasionally resulted in highly exaggerated curves. Consequently, 13 animals (of 180) were removed from the 10 datasets for the statistical analysis of slope because they possessed outlying slope values in excess of two standard deviations from the group mean slope value. These animals were distributed approximately evenly over all of the groups (AN19 in the

CON10 group, AN8, AN20 in the CON60 group, AN7 and AN18 in the PLM10 group, AN22 in the PLM60 group, AN11 in the PVC10 group, AN5 and AN13 in the PVC60 group, AN7 in the ALM group, AN15 in the ALM60 group, and AN3 and AN5 in the AVMALM60- group). This process produced a set of slopes for each group which were compared across groups with a two-way factorial (group-by-ISI) ANOVA. Three ANOVAs were run: One to compare initial slope of PLM- animals with controls, one to compare PVC- animals with controls and one to compare initial slopes of habituation for accelerations (on both ALM- and AVMALM- animals which lack head-touch mechanoreceptors). A one-tailed one-sample t-test was run to assess possible facilitation by determining whether the slope of the AVMALM- group was significantly larger than zero.

The asymptotic level of habituation was calculated by taking the mean percent initial value of the responses to the last three tap stimuli during habituation. Statistical comparisons for asymptote were the same as those described above for the comparison of initial slope.

Dishabituation was assessed by subtracting the asymptotic response level of habituation from the mean of the first three responses to tap after the application of a shock. This measure was referred to as the dishabituation difference score. A one-sample t-test was then used to assess dishabituation for each group. Dishabituation was deemed present if this score was significantly above zero. These findings have presented (Wicks & Rankin, in press-a).

Results

A central theme which emerged from the analysis of the circuitry underlying the tap withdrawal reflex (Wicks & Rankin, 1995a) was that the reflex is not unitary. The animal's net behavioural output in response to a tap stimulus appeared to be determined by a balance between the propensity to reverse—a behaviour initiated by stimulation of the anterior touch cells (ALM and AVM)—and the propensity to accelerate—a behaviour initiated by stimulation of the tail-

Figure 16: Increase in the number of accelerations (swimming forward) during habituation training at two ISIs. Intact sham operated animals trained with 40 tap stimuli at either a 10 s or a 60 s ISI demonstrated an increase in the frequency of accelerations during habituation training. The average number of accelerations per block of ten stimuli are shown for each group across training. Animals trained at a 10 s ISI also emitted more accelerations than did animals trained at a 60 s ISI. Error bars indicate the standard error of the mean.

BLOCK OF TEN STIMULI
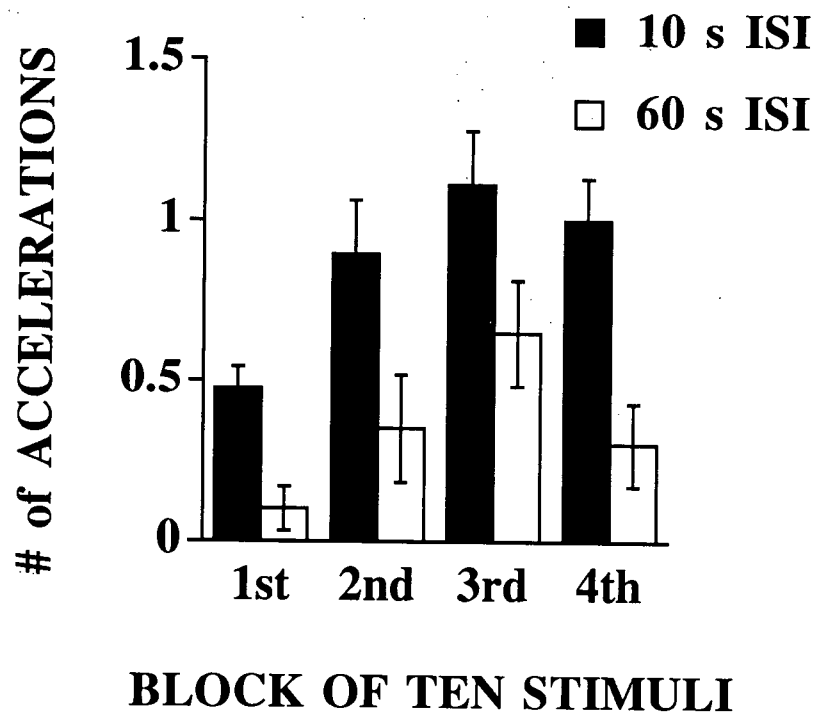
touch cells (PLM). In intact animals, the reversal response generally predominates (Rankin et al., 1990). Since it is possible to examine the contribution of each subcomponent of the reflex using laser ablation, we can determine whether the reversal reflex and the acceleration reflex both habituate, and if so, how the kinetics of habituation of these reflexes combine to determine the intact response as an animal learns. In this experiment, we first characterized habituation of both the reversal and the acceleration responses by training intact animals or animals which, due to ablations of neurons in the tap withdrawal circuit, either always reversed or always accelerated in response to tap. This assessment of habituation kinetics was done at two interstimulus intervals (ISIs).

Habituation.

Intact animals in the sham operated control groups trained with 40 stimuli at either a 10 s ISI (n = 19) or a 60 s ISI (n = 20) occasionally accelerated in response to tap during training. A repeated-measure ANOVA of the number of accelerations in each block of ten stimuli showed that the acceleration frequency increased with training, $F(3,111) = 3.229$, $p = 0.0252$ (see Figure 16). Furthermore, animals in the 10 s ISI group accelerated more frequently than did animals in the 60 s ISI group, $F(1,37) = 7.692$, $p = 0.0086$. Thus, for intact animals, the behavioural choice between forward locomotion and backward locomotion in response to a tap stimulus is modulated both by experience and the ISI used during training.

The kinetics of habituation of the reversal reflex was first analyzed in intact controls and in two groups which lacked specific neurons that caused them to reverse to tap: The PLM-animals lacked the posterior tail-touch receptors; the PVC- animals lacked the PVC interneurons and thus the electrical synaptic input from the PLM touch cells. This input is thought to be required for posterior mechanosensory-induced forward locomotion (see Figure 15). The standardized reversal magnitude during habituation in either the control (10 s ISI, n = 19; 60 s ISI,

Figure 17: Habituation of the reversal response. The effects of ablation on overall habituation kinetics of the reversal response in (A) intact animals, (B) in animals lacking either the posterior lateral microtubule neurons (PLM), and (C) the PVC interneurons are shown at both a 10 s ISI and 60 s ISI. Each habituation curve was standardized to the mean of the initial response magnitude for that group to facilitate cross-group comparisons of slope and asymptote. Best-fit curves (bf) were derived from the standardized curves by curve fitting individual animal habituation curves with a double exponential equation, and then extracting a group curve by averaging each animal's best-fit. The curves derived from the average of the best-fit curves for each animal demonstrated a very good fit to the group data and are shown superimposed on each groups standardized data. The PLM- animals differed from controls both in terms of initial rate of habituation and asymptotic level of habituation, whereas the PVC- was indistinguishable from controls on both of these measures. In all cases the reversal response to tap habituated more slowly at a 60 s ISI than at a 10 s ISI. See Figure 3 for response measurement procedure.
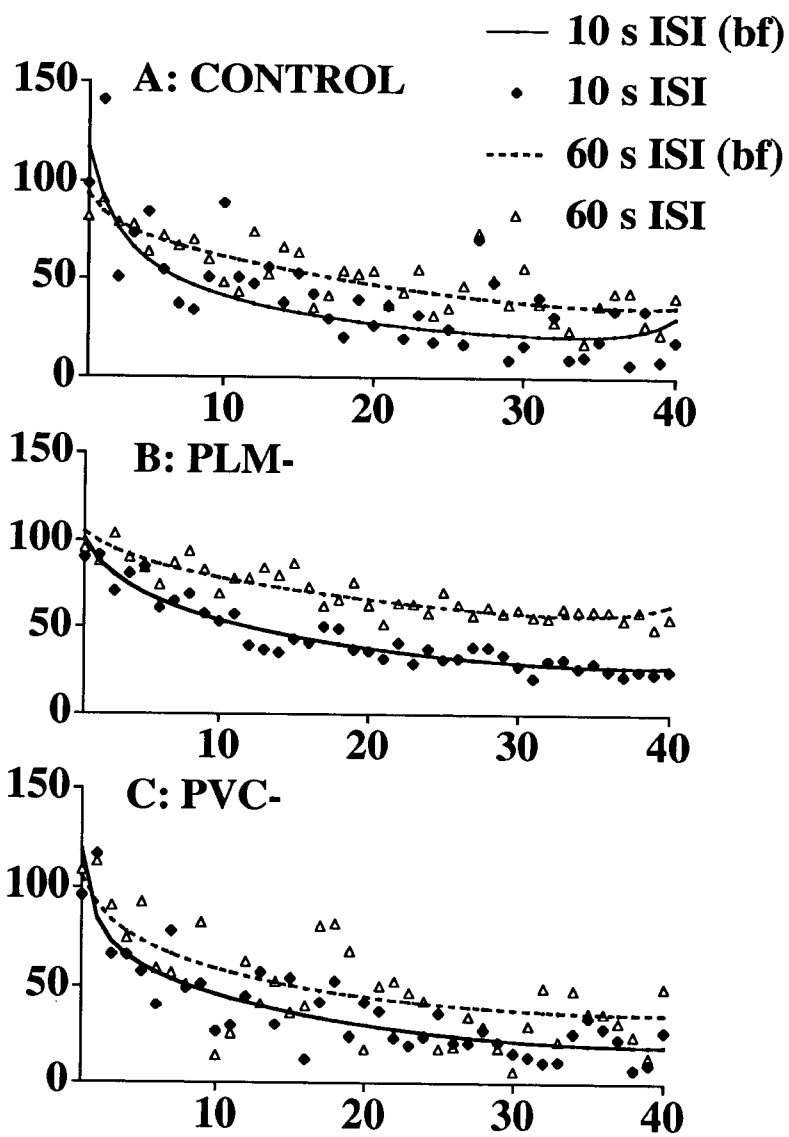
Figure 18: Habituation of the acceleration response. The effects of ablation on the overall

habituation kinetics of the acceleration response in animals lacking either (A) the anterior lateral

microtubule neurons (ALM) or (B) the anterior ventral microtubule neuron (AVM) in addition to

the ALM neurons are shown at both a 10 s ISI and 60 s ISI. These ablations result in acceleration

responses (swimming forward) to tap. Each habituation curve was standardized to the mean of

the initial response magnitude to facilitate cross-group comparisons of slope and asymptote.

Best-fit curves (bf) were derived as described in Figure 17 and are shown superimposed on each

group's standardized data. These data indicate that the acceleration response does habituate in

both groups. There is an initial facilitation followed by response decrement evident at a 10 s ISI.

No facilitation is observed at a 60 s ISI. See Figure 3 for response measurement procedure.
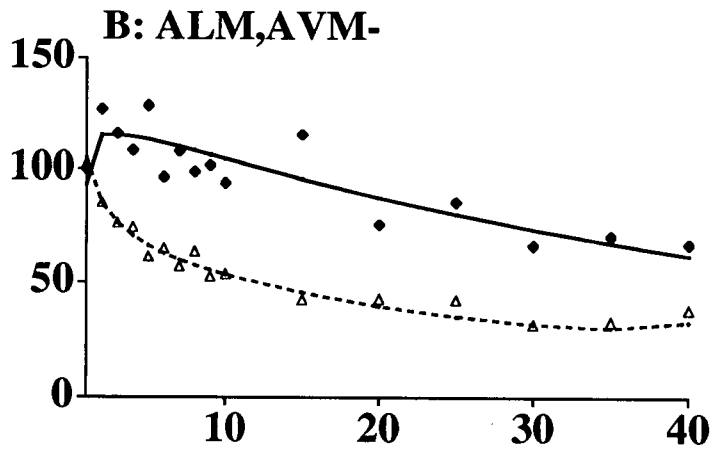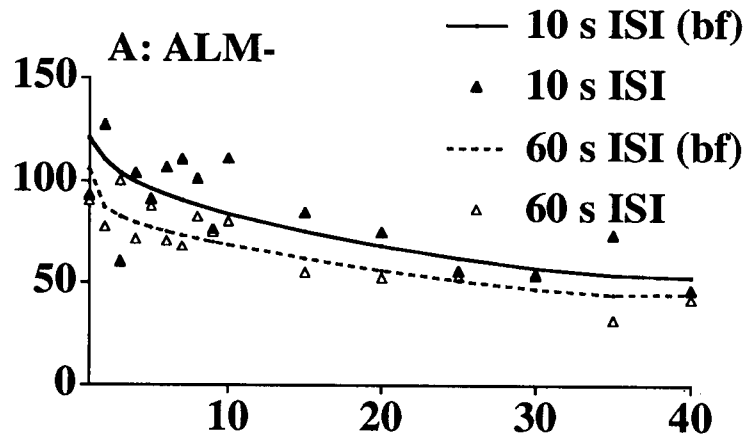
n = 20), PLM- (10 s ISI, n = 18; 60 s ISI, n = 22) or PVC- (10 s ISI, n = 19; 60 s ISI, n = 16) animals is shown in Figure 17. The best-fit curve for each group was derived and is superimposed on that group's data. The tap withdrawal response habituated both in the intact animals and in animals which lacked either the PLM tail-touch receptors or the PVC interneurons. Furthermore, one-tailed t-tests showed that all three groups habituated at both 10 s and 60 s ISIs (control, 10 s ISI, t = 8.5, p < 0.0001; control, 60 s ISI, t = 3.41, p = 0.002; PLM-, 10 s ISI, t = 6.54, p < 0.0001; PLM-, 60 s ISI, t = 3.90, p = 0.0005; PVC-, 10 s ISI, t = 5.84, p < 0.0001; PVC-, 60 s ISI, t = 1.81, p = 0.047).

Although during habituation training in intact animals accelerations were occasionally emitted, it was not possible to analyze them directly due to the low frequency of their occurrence. Therefore, the habituation of accelerations was analyzed by ablating the anterior touch cells which produced worms that predominantly accelerated in response to tap; no reversals were observed in this group of animals. The habituation curves for the acceleration response to tap observed in either ALM- (10 s ISI, n = 19; 60 s ISI, n = 24) or AVMALM- (10 s ISI, n = 13; 60 s ISI, n = 18) groups are shown in Figure 18. Again, the mean best-fit curves are superimposed on the habituation data for each group. Both of these ablation groups demonstrate that accelerations also habituated in response to repeated tap stimuli at both a 10 s and a 60 s ISI (ALM-, 10 s ISI, one-tailed t-test, t = 3.24, p = 0.003; ALM-, 60 s ISI, one-tailed t-test, t = 3.12, p = 0.003; AVMALM-, 10 s ISI, one-tailed t-test, t = 3.09, p = 0.005; AVMALM-, 60 s ISI, one-tailed t-test, t = 3.39, p = 0.003).

Initial rate of habituation

The initial rate of habituation for the reversal response was calculated from the best-fit curves (see Methods) and is shown in Figure 19A. The PLM- group showed a significantly smaller initial slope than did the control animals, $F(1,71)$ = 4.08, p = 0.047. There was a large

Figure 19: The initial rate of habituation. The initial rate of habituation for (A) reversals and (B) accelerations was derived explicitly by taking the mean of the initial slope of a best-fit curve for each animal. These slopes were then averaged and compared with an ANOVA. Since the data are standardized to the mean of the initial response magnitude, the slope is measured as a change in the percent initial response per stimuli. Comparisons made between PLM- and intact animals suggest that the PLM- animals habituated more slowly than controls; PVC- animals were not distinguishable from controls. The AVMALM- animals habituated more slowly than the ALM- animals, but this effect is largely ISI-dependent and is due to the facilitation of accelerations seen at a 10 s ISI. Error bars indicate the standard error of the mean.
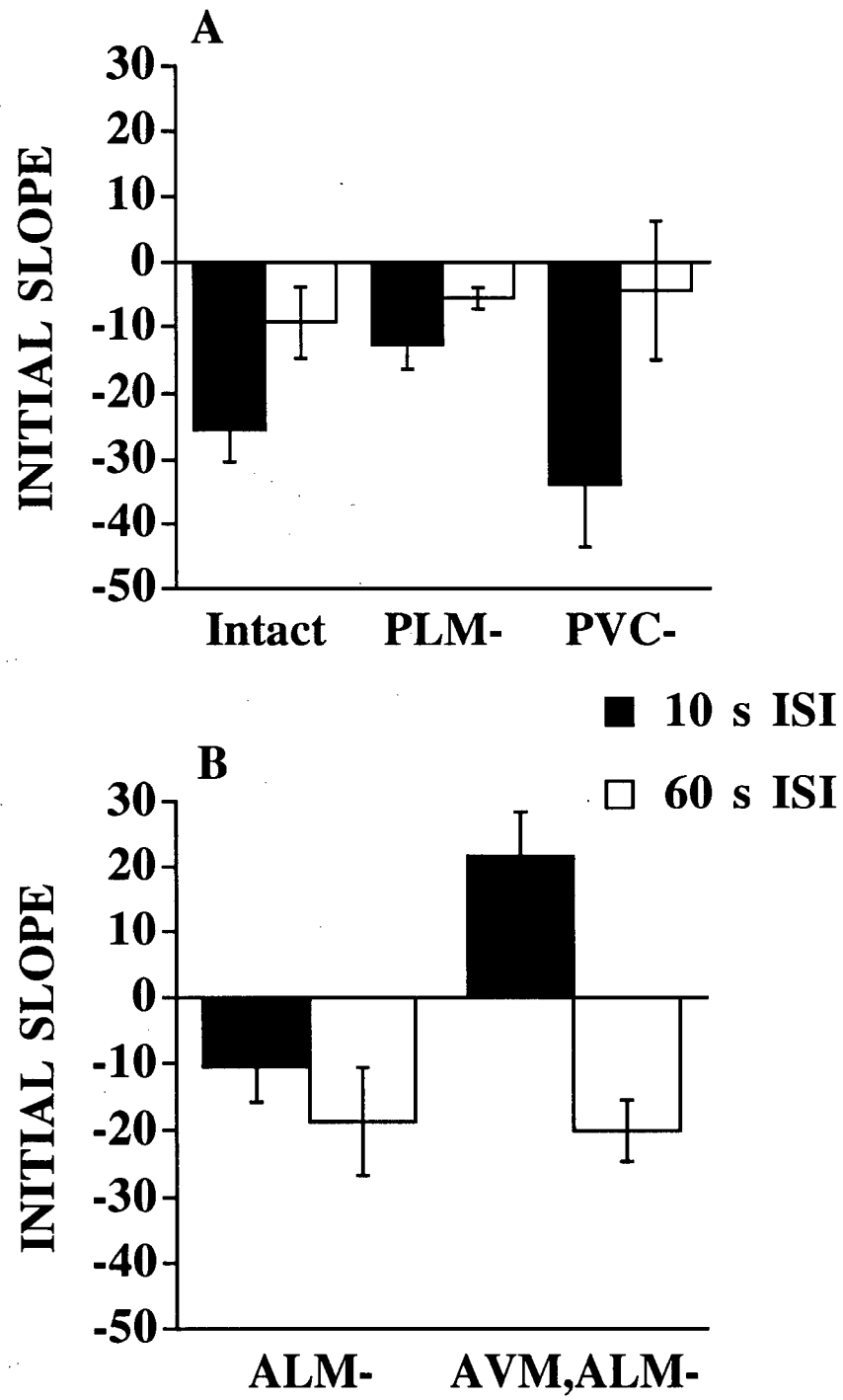
effect of ISI. Animals in both the PLM- group and the control group habituated more rapidly at a

10 s ISI than at a 60 s ISI, $F(1,71) = 8.38$, $p = 0.005$. A similar comparison of the control animals

with the PVC- animals again showed a more rapid habituation to a 10 s ISI than to a 60 s ISI,

$F(1,67) = 8.08$, $p = 0.006$, however there was no difference observed between control and PVC-

animals, $F(1,67) = 0.052$, $p = $ n.s.. Thus, animals had slower rates of habituation at a 60 s ISI

than at a 10 s ISI, and the PLM- animals had a slower rate of habituation than the control group,

whereas PVC- animals were indistinguishable from intact animals.

One important distinction between the PVC- and PLM- ablations—although certainly not

the only difference (see Discussion)—is that the PVC-,ablation leaves the chemical input from

the PLM touch cells onto the interneurons of the tap withdrawal circuit intact, whereas the PLM

ablation removes almost all input from the posterior mechanosensory field. Both ablations

remove the primary excitatory input to the forward locomotion circuitry and both PVC- and

PLM- animals are insensitive to light touch (Chalfie et al., 1985). Both Chalfie et al. (1985) and

Wicks and Rankin (1995a) have hypothesized that the chemical synapses from PLM have a

functional inhibitory effect on circuitry which controls backward locomotion. Thus, although

both ablations remove the primary excitation of forward mechanosensory-induced locomotion,

only the PVC ablation leaves the putative mechanosensory-induced inhibition of backward

locomotion intact. The finding that PVC- animals and controls habituated at the same rate, but

that PLM- animals habituated at a slower rate led us to hypothesize that the activity of the

chemical synapses from PLM to AVD, AVA and DVA (see Figure 13) might contribute to the

initial rate of habituation of the reversal response.

The initial rate of habituation was similarly assessed for the acceleration response in

ALM- and AVMALM- animals and is shown in Figure 19B. Ablations of either ALM alone or

ALM and AVM together result in animals that accelerate to a single stimulus (Wicks & Rankin,

1995a). The ALM- ablation removes some of the electrical input from the anterior

mechanosensory field which is thought to underlie anterior mechanosensory-induced backward

locomotion; the double ablation removes all of this input, and also removes the bulk of the

chemical input thought to inhibit posterior mechanosensory-induced forward locomotion.

Although the ALM neurons do make a few chemical synapses, the density of these connections

is approximately one-third that of those made by AVM (White et al., 1986). Thus, the double

ablation had a large effect on the chemical synaptic input to the interneuronal circuitry shown in

Figure 13 while the ALM- ablation left this input largely intact (see Figure 13). Although

statistical analysis suggested that the AVMALM- animals had a slower initial rate of habituation

than ALM- animals, $\underline{F}(1,65) = 5.02$, $\underline{p} = 0.029$, and that animals trained at a 10 s ISI habituated

more slowly than animals trained at a 60 s ISI, $\underline{F}(1,65) = 13.23$, $\underline{p} = 0.0005$, the significant group-

by-ISI interaction, $\underline{F}(1,65) = 5.97$, $\underline{p} = 0.017$, suggested that these effects were largely due to the

facilitation seen at a 10 s ISI in the AVMALM- group (see Figure 18B). This facilitation was

verified statistically, $\underline{t} = 3.15$, $\underline{p} = 0.004$, using a one-sample t-test which showed that the initial

slope of the AVMALM- group was significantly larger than zero. A visual inspection of the

habituation curve for the ALM- animals suggested that there may indeed have been some

facilitation at a 10 s ISI in this group as well (see Figure 18A). However, this effect was not

expressed as a positive initial slope in the habituation curve, probably because the third datapoint

in this curve was depressed with respect to the remainder of the dataset.

The animals in the two 10 s ISI groups (ALM- and AVMALM-) in this experiment both

habituated more slowly than the animals in the 60 s ISI groups. This is in contrast to the reversal

response groups (Intact, PLM- and PVC-) in which animals trained at a 10 s ISI habituated more

rapidly than animals trained at a 60 s ISI (see Figure 17).

Figure 20: The asymptotic level of habituation. The asymptotic level of habituation of (A) the reversal response and (B) the acceleration response was calculated by taking the mean of the last three responses to tap in the habituation series. The data are expressed as percent mean initial response levels. The PLM- animals had a higher asymptotic level of habituation than the intact controls whereas the PVC- animals were not different from the control animals. The acceleration measure demonstrated an interaction between the ablation group and ISI of training. The AVMALM- animals which lacked all anterior input to the tap withdrawal circuit were sensitive to the ISI of training and had a higher asymptotic level of responding at a 10 s ISI. This is consistent with a slower rate of habituation at a 10 s ISI shown in Figure 19B. The asymptotic level of responding in ALM- animals was not sensitive to ISI. Error bars indicate the standard error of the mean.
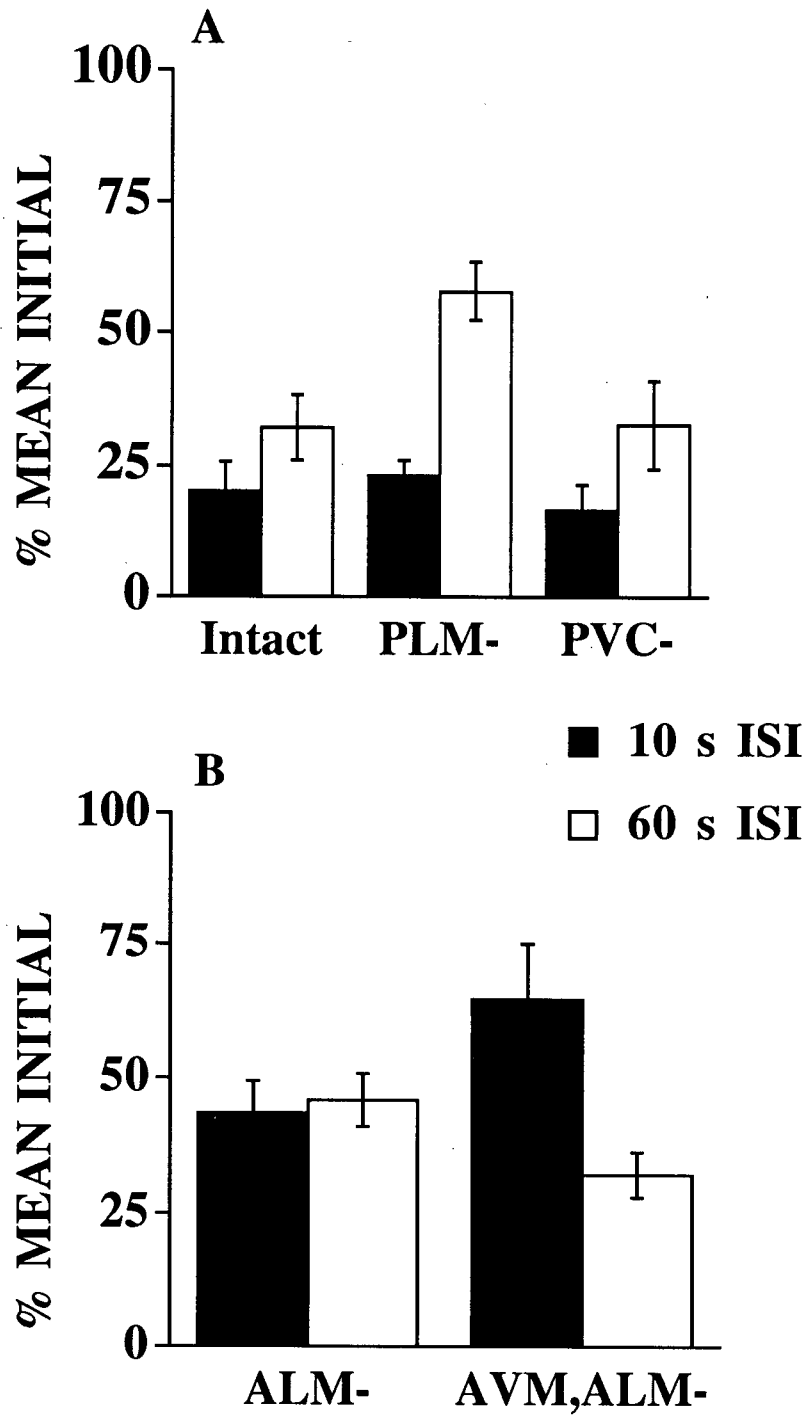
The asymptotic level of habituation.

The asymptotic response level for reversals was assessed by taking the mean of the last three responses to tap in the control, PLM-, and PVC- groups at a 10 s and a 60 s ISI and is shown in Figure 20A. A comparison of the intact animals with PLM- animals using an ANOVA suggested that PLM- animals had a higher asymptotic level of responding than controls (main effect of ablation, $F_{(1,76)} = 6.42$, $p = 0.013$), and that animals trained at a 60 s ISI had a higher asymptotic level of responding than did animals trained at a 10 s ISI (main effect of ISI, $F_{(1,76)} = 16.98$, $p < 0.0001$). This effect was larger for PLM- animals (ISI-by-ablation interaction, $F_{(1,76)} = 4.08$, $p = 0.047$), although both the main effects were supported by Fisher's PLSD post-hoc tests (ISI, $p < 0.0001$; ablation, $p = 0.007$). An ANOVA comparing the control animals to PVC- animals showed that the main effect of ISI was also significant in this comparison, $F_{(1,72)} = 4.96$, $p = 0.029$. However, there was no difference between control animals and PVC- animals, $F_{(1,72)} = 0.06$, $p = $ n.s., nor was there any significant ablation-by-ISI interaction, $F_{(1,72)} = 0.11$, $p = $ n.s.. Thus, control, PLM- and PVC- animals had higher asymptotic levels of responding at a 60 s ISI than at a 10 s ISI, and the PLM- had a higher asymptotic level of responding than the control group, whereas PVC- animals were indistinguishable from intact animals.

The asymptotic level of habituation of accelerations in ALM- and AVMALM- animals was assessed by taking the mean of the last three responses to tap and is shown in Figure 20B. Although there was an effect of ISI, $F_{(1,68)} = 5.71$, $p = 0.02$, the significant interaction, $F_{(1,68)} = 7.71$, $p = 0.007$, suggested that the ALMAVM- animals trained at a 60 s ISI possessed a larger asymptotic level of responding than AVMALM- animals trained at a 10 s ISI. There was no difference in the asymptotic level of habituation between ALM- animals trained at a 10 s ISI and ALM- animals trained at a 60 s ISI.

Dishabituation.

Thompson and Spencer (1966) listed dishabituation among the defining characteristics of habituation. It is a facilitation of a decremented or habituated response as a consequence of the application of a novel or noxious stimulus. Dishabituation has been studied extensively in Aplysia (Carew et al., 1971; Castellucci et al., 1970; Rankin & Carew, 1988).

Following habituation training, dishabituation was assessed by analyzing the magnitude of reversals evoked by tap stimuli after the application of an electric shock to the agar substrate upon which the animals moved. The mean of the last three responses prior to the shock was subtracted from the mean of the first three responses after shock. This measure was referred to as the dishabituation difference score. A one-sample t-test was then run on each group to determine whether that group had a difference score significantly above zero. The results are summarized in Figure 21. The intact animals showed significant dishabituation at both the 10 s ISI ($t = 1.91$, $p = 0.002$) and 60 s ISI ($t = 3.92$, $p = 0.001$). The PVC- animals showed a similar pattern at both a 10s ISI ($t = 3.14$, $p = 0.0027$) and a 60 s ISI ($t = 2.17$, $p = 0.022$). The PLM- animals only showed significant dishabituation at the 60 s ISI ($t = 2.13$, $p = 0.022$); there was no dishabituation in the PLM- 10 s ISI group ($t = 0.88$, $p = $ n.s.). Dishabituation of accelerations was similarly assessed. The ALM- animals showed significant dishabituation at both ISIs (10 s ISI, $t = 2.16$, $p = 0.023$; 60 s ISI, $t = 3.71$, $p = 0.0006$). The AVMALM- animals, like the PLM- animals, only showed significant dishabituation at the 60 s ISI ($t = 2.33$, $p = 0.017$); there was no dishabituation at the 10 s ISI ($t = 0.80$, $p = $ n.s.). Thus, for both the reversal and acceleration response, the dishabituation stimulus increased the response magnitude between 10 and 45 percent. This did not raise the response magnitude in any group to pre-habituation levels. However, responding in at least two groups (the PLM- 10 s ISI group and the ALM- 60 s ISI

Figure 21: Dishabituation. Dishabituation of the reversal response (A) and the acceleration response (B) was assessed by subtracting the responses to the last three taps during habituation training from the responses to the first three taps immediately after the application of an electrical shock to the substrate on which the animal was moving. The dishabituation difference score was analyzed with a one-tailed one-sample t-test for each group. All groups showed dishabituation at a 60 s ISI. However the PLM- and AVMALM- animals failed to demonstrate dishabituation at a 10 s ISI, whereas the other groups did. Error bars indicate the standard error of the mean.
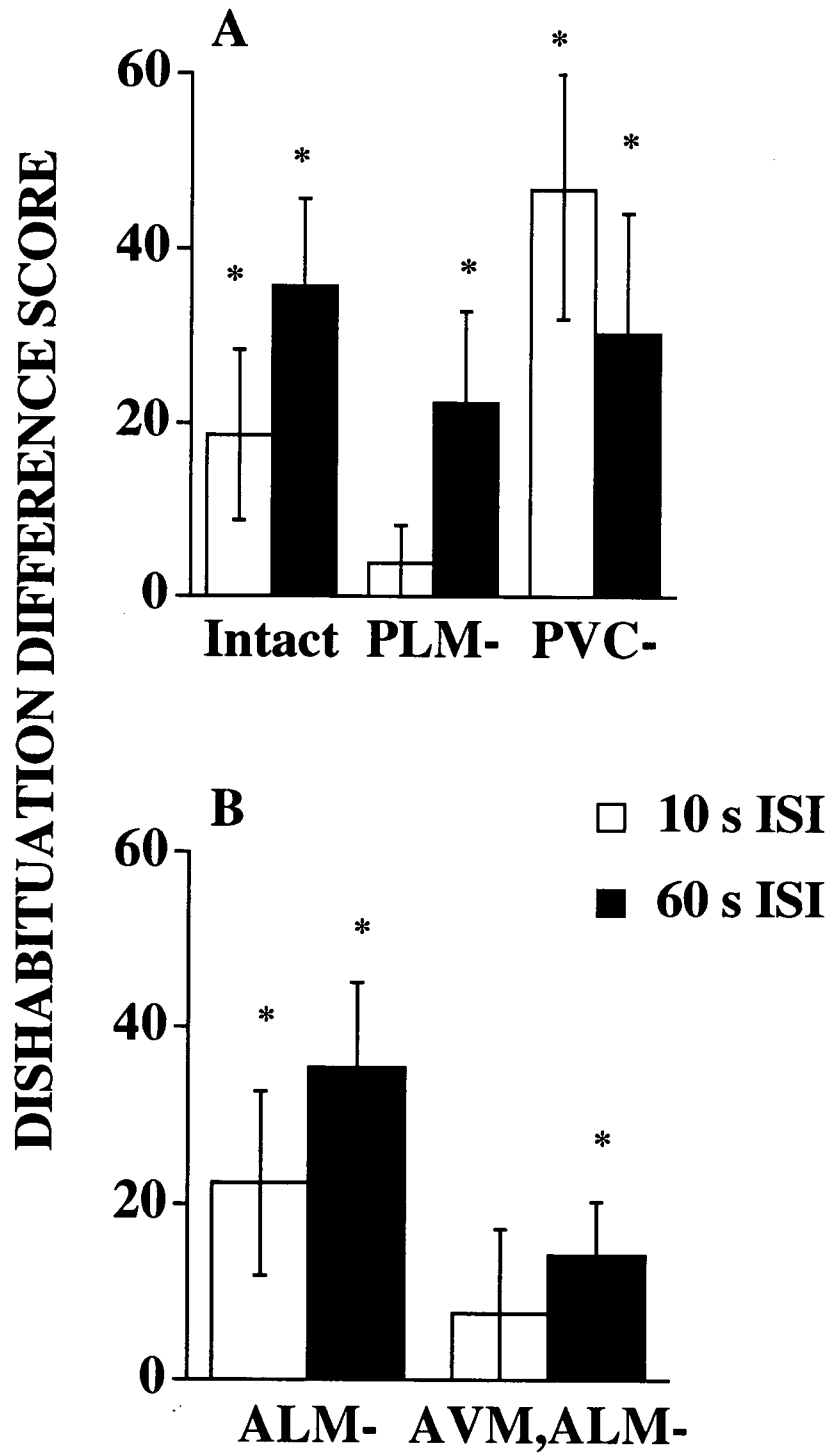
group) was measured at greater than 75 percent of pre-habituation levels (see Figures 16, 17 and 20).

This pattern of results suggested that the chemical synapses from the touch cells onto the interneurons of the tap withdrawal circuit (see Figure 13) are important for normal dishabituation. The cases of disrupted dishabituation involve those groups which had lost the largest fraction of these connections (PLM- and AVMALM-). Furthermore, dishabituation was disrupted only at a 10 s ISI in these two groups. This suggested that distinct processes may have been recruited during habituation at long and short ISIs. This is consistent with previous work which suggested that differential mechanisms, perhaps related to calcium influx, are recruited by long and short ISIs during habituation (Byrne, 1982; Klein & Kandel, 1980).

Discussion

Although the neural circuits that comprise the reflexive arc tend to be small, well defined mono- or poly-synaptic pathways, the neural populations that are activated by the stimuli used to elicit reflexes can be varied and complex (Falk et al., 1993; Kanz, Eberly, Cobbs & Pinsker, 1979; Peretz et al., 1976; Zecevic et al., 1989). Reflexes are the building blocks of more complex and integrated behaviours which might require the coherent action of several distinct reflexes simultaneously or in sequence. Additionally, a single circuit may mediate several reflexive behaviours depending on the nature of, or the context within which, a stimulus is applied (Getting, 1989; Selverston, 1988). In this paper we describe the relationship between the habituation of the nematode tap withdrawal reflex and the circuitry which underlies that reflex. Our results are interpreted within a framework, first described by Croll (1975), in which the control of nematode mechanosensory responding was characterized by the existence of two antagonistic central pattern generators: One for a "forward component" and one for a "reversal component". This framework is extended here to interpret changes in behaviour as a consequence

of repeated stimulation to be the result of changes in each of the underlying components of the intact behaviour.

Habituation kinetics were analyzed in five related groups of animals: Control animals possess intact tap withdrawal response circuitry; PLM- animals lack posterior mechanosensory input to the tap withdrawal circuit; PVC- animals lack the primary excitatory input from the PLM neurons but leave the putative inhibitory input from the PLM neurons intact; ALM- animals lack two of the three anterior neurons which transduce anterior mechanosensory input (but maintain the bulk of the putative inhibitory input from these cells onto the tap withdrawal circuit); AVMALM- animals lack all of the anterior microtubule neurons and thus all of the putative inhibition of forward locomotion. These groups allowed us to examine separately the intact behaviour as well as each of the two antagonistic responses that comprise the intact behaviour. These data are interpreted within the context of a hypothesis regarding the functional roles of the neurons and synapses of the tap withdrawal circuit. Specifically, it has been suggested that the chemical synaptic output of the PLM neurons functionally inhibits backward locomotion initiated by stimulation of the anterior touch cells. Similarly, the chemical synaptic output of AVM and ALM is thought to functionally inhibit forward locomotion initiated by stimulation of PLM (Chalfie et al., 1985; Wicks & Rankin, 1995a). Putative synaptic polarities for these and other neurons of the tap withdrawal circuit have been proposed on the basis of a computational model of this circuit (Wicks et al., 1996).

Two caveats must be stressed when using this lesion approach to localize and study plasticity in a circuit. First, the ablation of a neuron like PVC is likely to affect the complex dynamic properties of a feedback-laden network such as the tap withdrawal circuit in non-intuitive ways. Second, circuits are not static; a single physical circuit may possess several distinct functional states which are modulated by the neurochemical environment (Getting, 1989;

Harris-Warrick & Marder, 1991; Selverston, 1988). Although some aspects of C. elegans development show anatomical and functional compensation to the effects of laser ablation (Kimble, 1981; Kimble & White, 1981; Sulston & White, 1980), most neuronal cell classes lose function entirely if they are killed early in development (Avery & Horvitz, 1987; Avery & Horvitz, 1989; Bargmann & Horvitz, 1991), and no functional compensation has been noted for the neurons ablated in this report when these cells were killed early in development (Chalfie et al., 1985). Thus, circuit morphology was assumed to be unchanged by ablation or across the conditions within which these studies were run.

These results show that the two antagonistic responses to tap—acceleration and reversal—each habituate, and do so with distinct characteristics which can be reconciled with aspects of the changes in behaviour in intact animals during habituation (see Figure 22). For example, the differential effect of ISI on the initial slope of the habituation of the reversal response in intact animals (see Figure 17A) could be due to the fact that, at a 10 s ISI, the acceleration response shows an initial facilitation (see Figure 18). In an intact worm, this would be expressed as a fast initial rate of habituation of the intact reversal reflex at a 10 s ISI relative to a 60 s ISI, as the reversal subcomponent of the intact response received greater competition from the facilitated acceleration subcomponent at a short ISI. This would also explain why there is a higher overall number of accelerations seen at a 10 s ISI (see Figure 16). There are more accelerations seen at a 10 s ISI (than at a 60 s ISI) because the acceleration component decrements more slowly, and to a lesser extent than the reversal component at a 10 s ISI (see Figure 17 and 17). Additionally, the increase in the frequency of accelerations observed at a 10 s ISI (see Figure 16) is due to the fact that the acceleration subcomponent of the net behaviour habituates more slowly and relatively less robustly than the reversal subcomponent of the net behaviour at that ISI.

Figure 22: Habituation kinetics of two antagonistic responses. The reversal response and the

acceleration response each habituate, and do so with distinct rates. These kinetics are consistent

with habituation of the intact response.
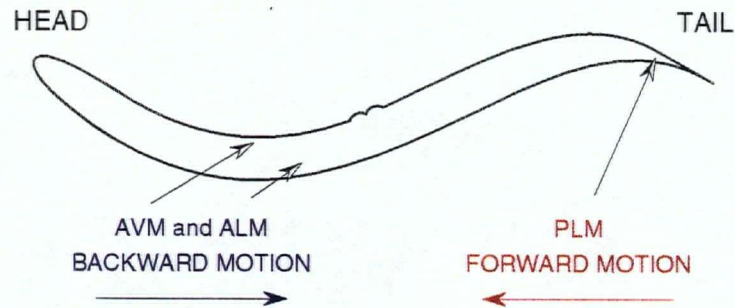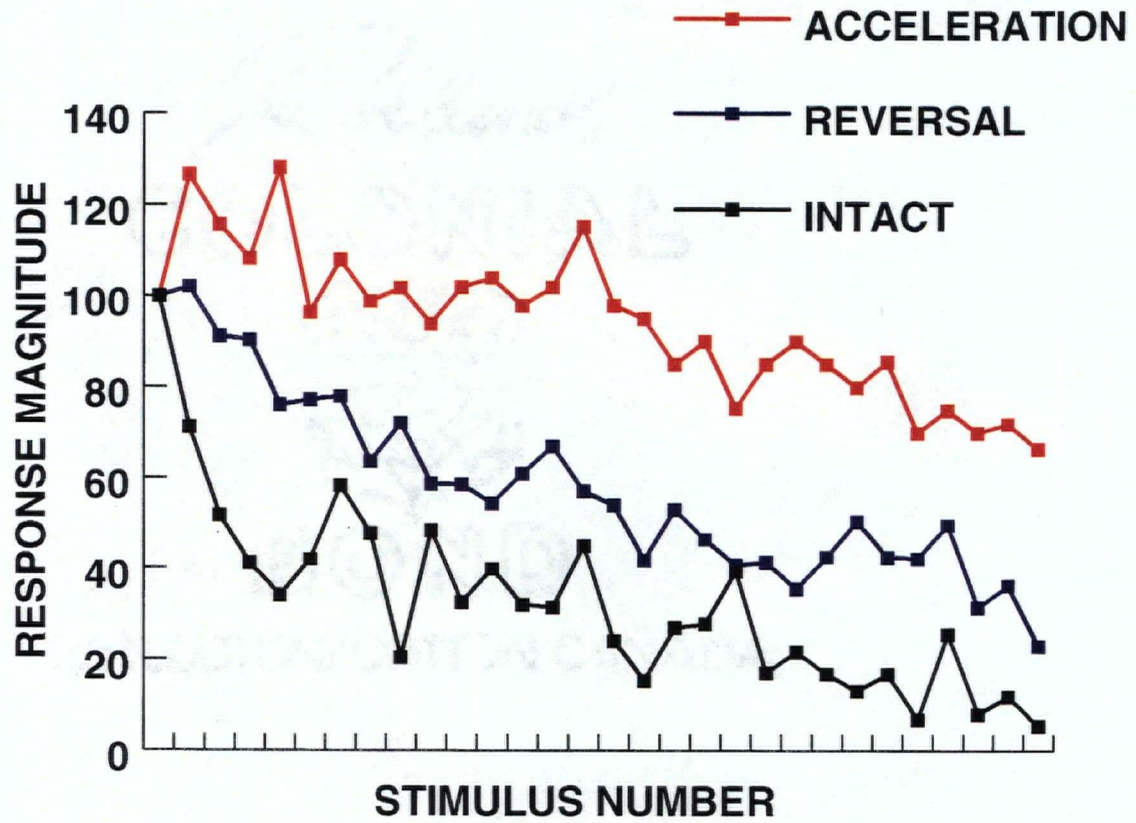
These results also emphasize the distinct effects of training at long (60 s) and short (10 s) ISIs. The dual-process theory of habituation (Groves & Thompson, 1970) suggests that both decrementing and facilitating processes might underlie the net behavioural output of an animal during habituation. These results support this theory by providing explicit evidence that both processes are elicited by the same stimulus during training; the reversal component of the behaviour habituates, while the acceleration component of the behaviour shows a facilitatory process superimposed upon habituation of that behaviour. These results further show that the facilitation process we observe is elicited differentially at short ISIs. Thompson and Spencer (1966) noted that animals trained at a shorter ISI will tend to habituate more quickly and deeply than animals trained at a longer ISI. These results suggest a mechanism by which this general parametric feature of habituation may be expressed in C. elegans. In the case of habituation of the reversal reflex in intact worms, the faster habituation at a short ISI appears to be at least partially due to the differential recruitment of a facilitatory subcomponent of an antagonistic behaviour. That is, the rapid decrease in the magnitude of the reversal reflex observed at a short ISI in the worm is partially due to a transient increase in the propensity to accelerate. Even though the acceleration response is not normally exhibited in intact worms during habituation, these results suggest that changes in the circuitry that mediates an acceleration to tap are reflected as a change in the kinetics of the antagonistic reversal behaviour. The facilitation of the acceleration response is not observed at a long ISI and consequently, the reversal response decrements more slowly. In addition, ISI has a differential effect on dishabituation in C. elegans. In ablation groups which do disrupt dishabituation (PLM- and AVMALM-), the disruption is only observed at a short ISI.

Finally, our results show that the ablations which disrupt chemical synapses from the mechanosensory neurons have a differential effect on the kinetics of habituation. This observation suggests that the integrity of these chemical connections is required for the

expression of at least some aspects of the plasticity which underlie habituation of the tap withdrawal response. The PVC- animals are indistinguishable from control animals in terms of response magnitude (Wicks & Rankin, 1995a), initial habituation rate (see Figure 19) and asymptotic level of habituation (see Figure 20), whereas the PLM- animals can be distinguished from controls on all three of these measures. Both the PLM- and PVC- ablations disrupt the primary excitation of forward locomotion; indeed, both ablations result in animals which are insensitive to light tail-touch (Chalfie et al., 1985) and reverse consistently (Wicks & Rankin, 1995a), yet the PVC- ablation leaves the chemical synaptic input from the PLM neurons intact (see Figure 13). In addition, both the PLM- ablation group and the AVMALM- ablation group, which disrupt the largest number of chemical synapses, show disrupted dishabituation at a 60 s ISI.

Although the neurotransmitter-receptor pairings, and hence the functional sign of these connections, is unknown, it has been speculated that, on the basis of the functional consequences of ablating these cells, these synapses are the source of functional inhibition of the antagonistic reflex circuitry (Chalfie et al., 1985; Wicks & Rankin, 1995a). This speculation has been supported by polarity predictions made for these synapses on the basis of a computational model of the tap withdrawal circuit (Wicks et al., 1996). The role of inhibition in the selection of behavioural output in reflexes that demonstrate habituation has been examined both empirically (Balaban, Bravarenko & Zakharov, 1993; Fischer & Carew, 1993; Kovac & Davis, 1977; Krasne & Teshiba, 1995; Rankin, 1991; Wicks & Rankin, 1991) and theoretically (Edwards, 1991; Ratner, 1970). Three potential roles for inhibition during habituation are: 1) habituation is a consequence of facilitation of inhibitory synapses, 2) habituation is a consequence of activity-dependent potentiation of recurrent inhibition, or 3) habituation is a consequence of decrement of inhibitory synapses, but it is the relative rate of decrement at sets of antagonistic synapses that

results in decrement of the overall response. As the worm tap withdrawal reflex is determined by the activity of antagonistic pathways, the results presented here are easily interpreted within the context of this last mechanism. However, these results also stress a role for facilitation in habituation of the tap withdrawal reflex.

We have demonstrated that habituation of the tap withdrawal reflex in C. elegans is not a unitary phenomenon. Rather, decrement of behavioural output is a consequence of two competing behaviours, each changing with distinct kinetics. The remarkable simplicity of the nematode nervous system has allowed us to characterize and localize these changes within a completely identified circuit.

Experiment 4: The Multiplicity of ISI-Dependent Processes

Thompson and Spencer (1966) have described a number of parametric criteria by which

habituation can be distinguished from other forms of response decrement. These criteria include

two major effects of the interstimulus interval (ISI) used during training. First, the kinetics of the

habituation curve (i.e., initial rate and asymptotic level of habituation) are sensitive to ISI;

animals trained at a shorter ISI will habituate faster and deeper than animals trained at a longer

ISI. Second, the kinetics of recovery from habituation are also sensitive to the ISI used during

training; animals trained at a shorter ISI will recover from habituation faster than animals trained

at a longer ISI. This second effect is somewhat paradoxical in that the animals which recover

fastest are generally the same as those which appeared to be most habituated by the criteria of

habituation rate or asymptote.

This interpretation—that the kinetics of recovery are determined by the ISI used during

training—has remained unchallenged since it was introduced almost three decades ago, despite the

existence of an obvious alternative hypothesis. It is also possible that the kinetics of recovery

from habituation are determined by intrinsic properties of the habituation curve itself rather than

the ISI per se. That is, the behavioural consequences of habituation at a given ISI (i.e., rate and

asymptotic level of habituation) might determine the kinetics of recovery from habituation.

However, since ISI also determines the kinetics of habituation, it has not been possible to address

this question adequately.

In this study, we utilize single-cell microsurgical ablations of the PLM tail-touch

receptors to manipulate the rate and degree of habituation of the tap withdrawal reflex in C.

elegans. The intent of this experiment was to determine whether the kinetics of recovery from

habituation of this reflex are correlated with the ISI used during habituation training, or alternatively, by the kinetics—that is, the rate and depth—of habituation, independent of the ISI.

Materials and Methods

Subjects.

A total of 60 hermaphroditic N2 Caenorhabditis elegans were used.

Procedure.

Single-cell laser ablations were performed as described previously (Wicks & Rankin, 1995a) in three groups of animals. Three groups of worms (CON10, CON60 and PLM-) underwent either a mock-surgical procedure or bilateral removal of the PLM- neurons, which resulted in animals which consistently reversed to tap. Individual animals were raised on separate plates and tested for habituation 4 days post hatching.

Habituation training was conducted as described in Rankin et al. (1990) with the following modifications. Animals were raised on individual plates seeded with E. coli in this study, and transferred to unseeded testing plates just prior to testing. Individual plates were placed gently under the optics of the dissecting microscope and the worm was allowed to recover for 1 to 2 min. Then, 30 tap stimuli were delivered to the plates at either 10 or 60 s intervals and the behaviour was recorded. Recovery stimuli were delivered to animals following intervals of 30 s, 5 min, 10 min and 20 min after the last habituation stimulus.

Analysis.

Statistical comparisons of slope, asymptote and recovery were made using ANOVAs with Fisher's protected least significant difference (PLSD) planned comparisons where appropriate.

Two dependent measures were used to characterize the shape of a habituation curve: Initial rate and asymptotic level. We standardized all data such that the mean of the initial

response magnitude of each group was equal to one hundred percent since we were concerned particularly with comparing rates of habituation between groups with different initial levels of responding (Wicks & Rankin, 1995a). Curve-fitting was then used to determine the initial rate of habituation of each group. Five animals (of 60) were removed from the three datasets for the statistical analysis of slope; they possessed outlying slope values in excess of two standard deviations from the group mean slope value. A mean initial rate of habituation was derived for each group and compared using a factorial ANOVA. The asymptotic level of habituation was calculated by taking the mean percent-initial value of the responses to the last three tap stimuli during habituation and analyzed with a factorial ANOVA.

These findings have been presented in published form (Wicks & Rankin, in press-b).

<u>Results</u>

The habituation curves obtained from the three groups are shown in Figure 23A. Two groups of intact animals were trained at either a 10 s ISI (CON10, n = 19) or a 60 s ISI (CON60, n = 18). A third group of worms (PLM10, n = 23) underwent surgical ablation of the tail-touch receptors and were habituated at a 10 s ISI. Visual inspection of the data suggest that habituation curve kinetics are sensitive to both ISI and lesion, which replicates earlier findings (Rankin & Broster, 1992; Wicks & Rankin, in press-a).

The analysis of initial rate of habituation was conducted on best-fit curves shown in Figure 23B. A factorial ANOVA of the initial slopes of the three best-fit curves indicated that there was a significant effect of GROUP, $\underline{F}(2,52) = 4.1$, $\underline{p} < .05$. Fisher's PLSD further indicated that the group initial slope PLM10 (mean = -6.8, SE = 2.4) was significantly shallower than the initial slope of the CON10 (mean = -30.0, SE = 8.8) group, Mean Difference = -23.1, Critical Difference = 16.4, $\underline{p} < .01$, whereas the PLM10 group was not significantly different from the CON60 group (mean = -20.2, SE = 5.5), Mean Difference = -13.4, Critical Difference = 16.9, $\underline{p} >$

.1. Thus, the PLM- animals trained at a 10 s ISI habituated slower than control animals trained at a 10 s ISI and habituated at the same rate as the control animals trained at a 60 s ISI.

The analysis of the asymptotic level of habituation echoed this pattern of results. A factorial ANOVA on the mean of the last three responses of each group indicated that the asymptotic level of responding was sensitive to GROUP, $F(2,57) = 12.3$, $p < .0001$. Furthermore, Fisher's PLSD indicated that the asymptotic level of responding of the PLM10 group (mean = 30.7, SE = 4.8) was higher than the CON10 group (mean = 7.1, SE = 2.9), Mean Difference = -23.6, Critical Difference = 12.9, $p = .005$, and was not different from the asymptotic level of responding of the CON60 group (mean = 39.6, SE = 5.7), Mean Difference = 8.9, Critical Difference = 13.1, $p > .1$. Thus, both with respect to initial rate and asymptotic level of habituation, animals that have received PLM ablations and are habituated at a 10 s ISI are indistinguishable from control animals trained at a 60 s ISI and are significantly different from control animals trained at a 10 s ISI.

The recovery points (30 s, 5 min, 10 min and 20 min) for the three groups are shown in Figure 23C. To determine whether the kinetics of recovery of the PLM- animals are correlated with the habituation kinetics or ISI, an ANOVA was run with a repeated-measures factor, RECOVERY (four levels, 30 s, 5 min, 10 min, and 20 min), and a between subject factor, GROUP (three levels, CON10, CON60 and PLM10). This analysis indicated that there was a main effect of GROUP, $F(2,46) = 6.0$, $p = .005$, and a main effect of recovery, $F(3,138) = 3.7$, $p < .05$, but there was no interaction between GROUP and RECOVERY, $F(6,138) = 0.4$, $p > 0.1$. Since there was no significant interaction in this analysis, the main effect of group was further analyzed with Fisher's PLSD which indicated that the PLM10 group was indistinguishable from the CON10 group, Mean Difference = 2.5, Critical Difference = 23.9, $p > .1$, and different from

Figure 23: Summary of the effects of ISI (10 s vs 60 s) and ablation (control vs PLM) on the

kinetics of habituation (A, B) and recovery (C) in three groups of animals. Asymptotic levels of

habituation were calculated as the mean of the last three responses of habituation curves

standardized to initial response levels (A) whereas the initial rate of habituation was calculated

from the mean initial slope of the best-fit curves (B) derived from the standardized data. The

animals with PLM lesions which were trained at a 10 s ISI (PLM10) were indistinguishable from

control animals trained at a 60 s ISI (CON60) on both measures, and significantly different from

control animals trained at a 10 s ISI (CON10). In contrast, the pattern of recovery (C) of the

PLM10 animals, expressed as the percent increase over asymptote, was indistinguishable from

CON10 animals and different from the CON60 animals. Error bars indicate SE. See Figure 3 for

response measurement procedure.
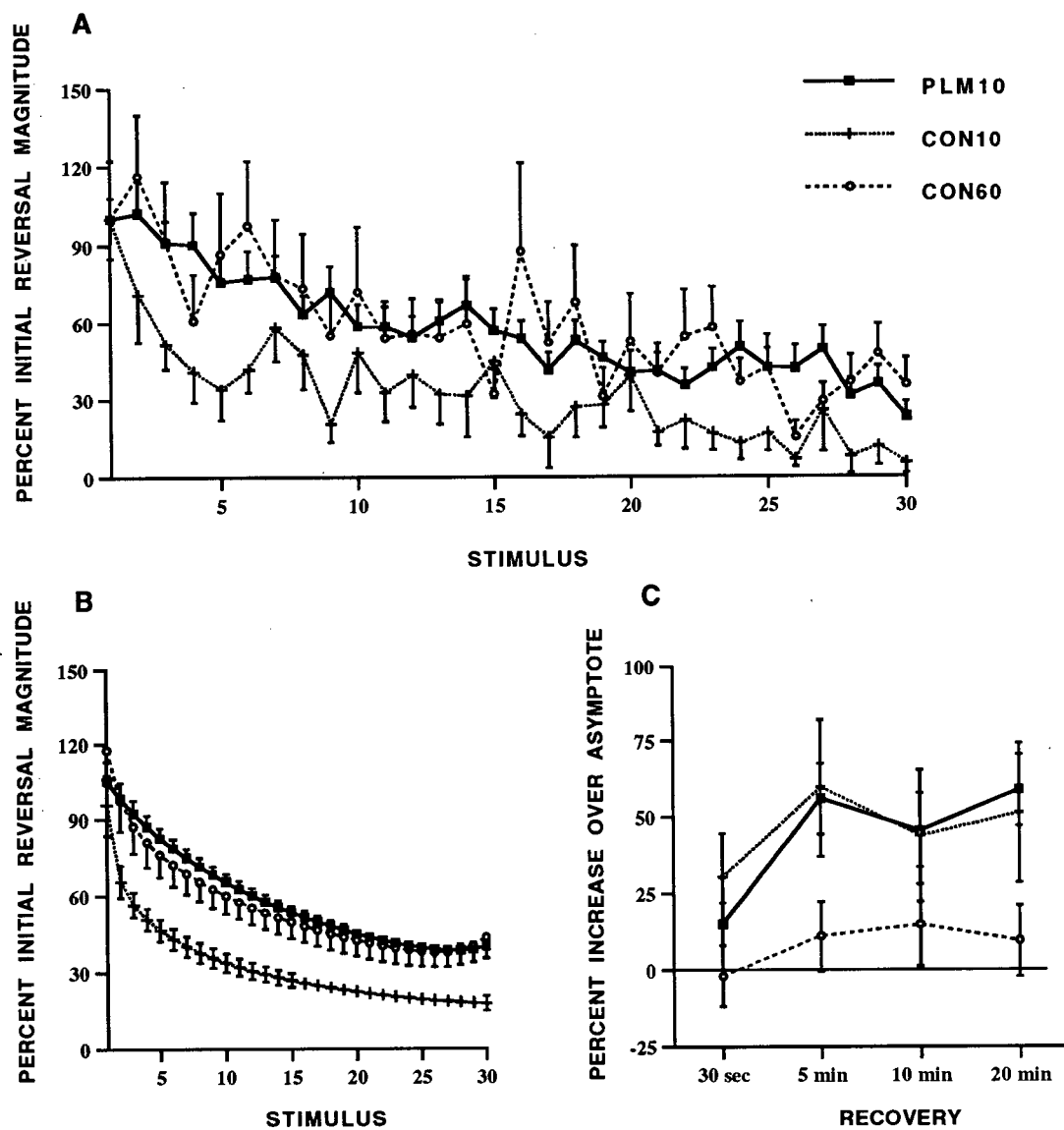
the CON60 group, Mean Difference = -35.5, Critical Difference = 22.8, $\underline{p}$ < .005. Thus, the kinetics of recovery are correlated with the ISI used during training rather than the intrinsic properties of the habituation curve.

Discussion

These results demonstrate explicitly for the first time that the kinetics of recovery from habituation are not determined by the behavioural consequences of training at a given ISI—that is, the rate or depth of habituation—but rather are dictated by ISI-dependent processes which don't impact directly upon behaviour during habituation training. Animals lacking the PLM tail-touch receptors which were trained at a short ISI and which habituated—as a consequence of the lesion—with kinetics that resembled habituation at a long ISI, nonetheless demonstrated a pattern of recovery which was similar to that of intact animals trained at a short ISI. This result suggests that at least two sets of ISI-dependent processes must be recruited during habituation training, one affecting habituation and one affecting recovery from habituation.

The analysis of the effects of ISI on recovery from habituation training has been carried out previously in several preparations, including the startle response of the rat (Davis, 1970), the Aplysia gill withdrawal reflex (Byrne, 1982), and the nematode tap withdrawal reflex (Broster & Rankin, 1994; Rankin & Broster, 1992). Several previous reports suggest that short ISIs recruit a facilitatory process which counters the classic decrementing process suggested by habituation. For example, Byrne (1982) has suggested that a simple neurotransmitter depletion model is insufficient to explain synaptic depression in Aplysia neurons and, consequently, Gingrich and Byrne (1985) have developed a model of synaptic depression which invokes calcium loading during stimulation at a high frequency as a facilitating process. Such a model is consistent both with theoretical considerations (Groves & Thompson, 1970; Ratner, 1970) and data presented

both here regarding recovery from habituation and previously with respect to rate of habituation

(Wicks & Rankin, in press-a; see Experiment 3) in <u>C. elegans</u>.

Experiment 5: The Localization of Habituation

Habituation—a simple form of learning—is often expressed as a decrement in a reflexive behaviour (Groves & Thompson, 1970; Sokolov, 1963; Thompson & Spencer, 1966). The physiological substrate of reflex habituation may be localized within the circuitry that mediates that reflex. For example, in the marine mollusk Aplysia , depletion of available transmitter at the sensory-motor neuron terminals is correlated with habituation of the gill withdrawal reflex (Bailey & Chen, 1988; Byrne, 1982). Alternatively, in some systems habituation is localized outside the reflex circuitry (Fischer & Carew, 1993; Krasne & Teshiba, 1995; Ratner, 1970). Even in Aplysia, analyses of plasticity within identified circuitry suggest that there are multiple sites which show experience-dependent changes in activity in addition to the monosynaptic component (Falk et al., 1993; Kanz et al., 1979; Peretz et al., 1976).

Habituation of the intact tap withdrawal response might be due to a change specific to the head-touch pathway, the tail-touch pathway, or both. Alternatively, the neural correlates of habituation might be localized to motoneuron pools or an interneuronal site downstream of, and common to, both the head-touch and tail-touch channels. In Experiment 3, it was shown that each component of the intact behavioural response is subject to habituation. For example, animals in which the tail-touch receptors have been removed by ablation and, as a consequence, lack input from the posterior mechanosensory pathway, consistently reverse to tap; with repeated stimulation, this response habituates (Wicks & Rankin, in press-b). A similar result was obtained for animals that consistently accelerate to tap as a consequence of the removal of the tail-touch cells; accelerations also habituate as a consequence of repeated stimulation. This suggests that the locus of change in the nervous system is not restricted to one class of sensory neuron, but does

not further disambiguate the locus of habituation; distributed sensory neuron, interneuron and motoneuron sites remain possible loci of change.

Elements of the tap withdrawal circuitry are involved in behaviours that are not evoked by a tap stimulus. Specifically, the motor neuron pools and at least two of the interneurons (AVA and AVB) form the CPG for locomotion (Chalfie et al., 1985). Thus, spontaneous reversal behaviour and reversal behaviour evoked by stimuli other than a tap each recruit the activity of these elements. The current studies were designed to investigate the loci of plasticity correlated with the habituation of the tap withdrawal response by analyzing the effects of the habituation of that reflex on the magnitude and frequency of reversal responses that are either emitted spontaneously or are induced with a thermal stimulus. If habituation of the tap withdrawal response results in a decrement in either of these behaviors, then plasticity may be localized to neural elements that are shared by each affected behavior. Alternatively, if habituation of the tap withdrawal response has no effect on either alternative behavior, then plasticity can be localized to the exclusive set of tap withdrawal response circuitry.

These findings has been presented in abstract form (Wicks & Rankin, 1995b) and have been submitted for publication (Wicks & Rankin, submitted).

<u>Experiment 5A</u>

Croll (1975) suggested that the behavioural output of a worm was a balance between a "forward component" and a "reversal component". He hypothesized that a reversal might be emitted when the strength of the "forward component" was reduced by either sensory input in the case of an evoked reversal or an endogenous decay process in the case of a spontaneous reversal. Croll characterized the nature of both spontaneous and touch-induced reversal behaviours in <u>C. elegans</u>. Both behaviours consist of a small, variable number of forward going sinusoidal waves, resulting in backward locomotion. However, the spontaneous reversal

behaviour is often terminated by an "omega" wave in which an animal's head and tail come into close proximity as the animal begins forward locomotion in a new direction. This is in contrast to reversals evoked by mechanosensory input to the nose (i.e., if the worm bumps into a glass bead), which are rarely followed by a significant change in heading.

Since nose touch-induced and spontaneous reversals appear to consist of somewhat distinct motor patterns, it was necessary to determine which of these motor patterns was induced by the tap stimulus. To determine if the tap stimulus was invoking the CPG that controls spontaneous reversal behaviour in the worm, the relationship between spontaneous reversal activity and tap-induced reversal activity in the adult animal was examined. Specifically, tap-induced changes in heading were compared to the data of Croll (1975) to determine if the tap response had a morphology more like that of a spontaneous or nose touch-induced reversal.

Subjects.

A total of 50 hermaphroditic N2 Caenorhabditis elegans were used.

Methods.

Individual animals were transferred from colony plates to foodless testing plates and allowed to recover from the transfer for at least 1 min. Then, a single tap stimulus was applied, and the heading taken by the animal after reversing was measured. Heading measures were only taken when, as a consequence of the tap stimulus, animals reversed. A total of 44 observations were made from the 50 animals used.

Scoring.

Heading measures were made by arbitrarily setting the heading of the animal along its body axis as 0 degrees immediately prior to the application of a tap stimulus. The post-tap heading (in degrees) was measured with respect to this pre-tap heading using a transparent

acetate overlay which was partitioned into segments of 30 degrees. Any change in heading was grouped into 30 degree bins according to the methods of Croll (1975).
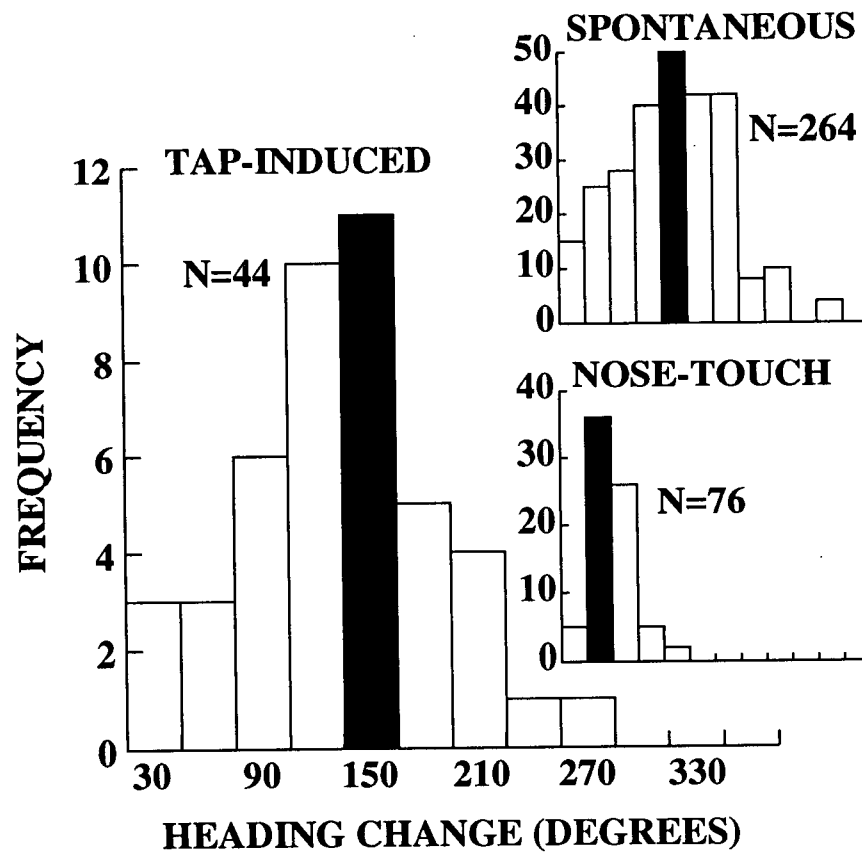
Analysis.

The frequency distribution of tap-induced heading changes was graphed and the modal post-tap heading compared to previously published data (Croll, 1975). To determine if the frequency distribution obtained in this experiment was consistent with the frequency distribution of heading changes associated with either nose touch-induced or spontaneous reversals, the data was compared to similar data from Croll's study using a Chi-squared analysis.

Results.

The frequency distribution of tap-induced heading changes is shown in Figure 24. Croll (1975) reported that modal change in heading of a worm after the emission of a spontaneous reversal was 150 degrees. In contrast, the modal change in heading of a worm after nose-touch stimulation was 60 degrees (see inset Figure 24). The modal change in heading associated with a tap-induced reversal was 150 degrees.

To determine if this distribution of heading changes was consistent with nose touch-induced heading changes, the data were compared to the results of Croll (1975) regarding heading changes as a consequence of spontaneous reversals or reversals induced by bumping into glass beads spread over the agar surface. Since the distribution of heading changes induced by bumping into beads reported by Croll had only one instance (in 74 observations) of a heading change in excess of 180 degrees, all bins of greater than 180 degrees were collapsed for this analysis as recommended by Siegel (1956). The null hypothesis in this analysis was that both tap-induced changes in heading and nose-touch induced changes in heading were drawn from the same population. This hypothesis was rejected, Chi-squared = 52.65, df = 5, p < .0001. Thus, the

Figure 24: Changes in heading as a consequence of the application of a tap stimulus. In response to tap, worms frequently exhibit an "omega" waveform, in which head and tail come together at the termination of a reversal response. This omega wave, which is also observed at the termination of spontaneous reversals, generally results in a substantial change in direction as a consequence of the reversal. This is contrasted with reversals evoked by the worm swimming forward into a glass bead which are followed by a modal change in heading of approximately 60 degrees (Croll, 1975).

distribution of heading changes evoked by nose touch is significantly different from that evoked by the tap stimulus; the responses have distinct shapes.

A similar analysis was run to compare the frequency distribution from Figure 24 with the distribution of heading changes associated with spontaneous reversal activity reported by Croll (1975). In this case, it was necessary to collapse all heading changes in excess of 270 degrees into a single bin for the analysis. In this comparison, the null hypothesis that the two samples were drawn from the same distribution was not rejected, Chi-squared = 5.191, df = 8, $\underline{p}$ = 0.737. Thus the frequency distribution of heading changes associated with spontaneous reversals and the frequency distribution of heading changes associated with tap-induced reversals are indistinguishable.

Experiment 5B

Experiment 5A demonstrated that both spontaneous reversals and tap-induced reversals share a similar morphology. There is also good anatomical evidence that the neural substrate of a spontaneous reversal and a tap-evoked reversal are largely overlapping, each including at least the motor neurons and the AVB and AVA interneurons. Only five interneurons (PVC, AVD, AVB, AVA and AVE) in the animal make extensive connections with the motor neurons which control locomotion in C. elegans. These all are part of the tap withdrawal circuit (Wicks & Rankin, 1995a) except AVE which makes synaptic contact only with the anterior portion of the ventral cord (White et al., 1986). Ablations of either the AVB or the AVA interneurons result in animals that are incapable of coherent backward and forward locomotion respectively, either spontaneously or in response to mechanosensory stimulation, whereas ablations of PVC or AVD leave locomotion intact (Chalfie et al., 1985; Wicks & Rankin, 1995a).

To determine whether the touch cells (ALM, AVM or PLM) or the mechanosensory-connector interneurons (PVC and AVD) played a role in initiating or maintaining spontaneous

reversal activity, the spontaneous reversal activity of animals either lacking all of the touch cells or lacking the PVC interneuron was compared to that of ablation control animals. Previous observations noted that, although the movement of animals lacking AVA, AVB or the motor neurons was severely disturbed (Chalfie et al., 1985; Wicks & Rankin, 1995), the movement of either PVC- or AVD-ablated animals was regular and appeared normal (although the tap withdrawal response magnitude was affected by the ablation). However, these observations focused on evoked responding rather than spontaneous behaviour. To evaluate the potential contribution of the PVC and AVD neurons to the production of spontaneous locomotion, only animals lacking the PVC neuron were prepared. The two neurons (PVC and AVD) have been shown to play parallel roles in the integration of forward and backward locomotion, and the position of AVD (adjacent to the basement membrane of the pharynx deep in the anterior ganglion) makes the unambiguous ablation of that neuron very difficult.

Subjects.

A total of 24 hermaphroditic N_2 Caenorhabditis elegans were used.

Methods.

All animals were isolated at hatching and prepared for laser microsurgery as described in the general methods. Three groups of animals were prepared: PVC- (n = 10), ALM,AVM,PLM- (n = 4) and CONTROL (n = 11). Individual animals were transferred from colony plates to foodless testing plates and allowed to recover from the transfer for at least one minute. Then, four minutes of spontaneous behaviour was recorded and the magnitude of all spontaneous reversals was measured.

Scoring.

Spontaneous reversals were measured using the methods described for the measurement of tap-evoked reversals.

Analysis.

An ANOVA was run on the magnitude of spontaneous reversals observed in the three

groups and Fisher's PLSD were used to assess differences between the groups. Rarely, very large

spontaneous reversals were emitted by individual worms in all three groups. These observations

increased the variance substantially, and so any reversal that was more than two standard

deviations above the group mean was removed from the dataset for the analysis. These were

distributed roughly evenly across the three groups (6 of 130 observations in the PVC- group; 6 of

98 observations in the CONTROL group and 1 of 35 observations in the ALM,AVM,PLM-

group). The number of reversals was analysed by expressing the data in terms of number of

reversals per animal and comparing each of the two ablation groups to the control condition with

an unpaired t-test.

Results.

Animals in all three groups demonstrated intact locomotion after transfer to testing plates.

The magnitude and frequency of spontaneous reversal activity in the three groups are shown in

Figure 25. No difference in the frequency or magnitude of spontaneous reversal responses

between the CONTROL group and the group of animals lacking all three touch cells

(ALM,AVM,PLM-) was observed. However, reversals emitted by PVC- animals tended to occur

more frequently than CONTROL animals, t = 2.37, df = 19, p = 0.028. Furthermore, the overall

ANOVA on spontaneous reversal magnitude indicated a significant difference between the

groups, F(2, 247) = 10.62, p < 0.0001. Fisher's PLSD suggested that the only significant

difference was between the CONTROL animals and the PVC- animals (see Figure 25). The

reversals emitted by the PVC- animals were smaller than those emitted by control animals,

MEAN DIFFERENCE = 116.6, CRITICAL DIFFERENCE = 50.2, p < 0.0001.

Figure 25: The effects of laser ablation on the magnitude (A) and frequency (B) of spontaneous

reversal behaviour is shown for three groups. Error bars indicate standard error of the mean. See

Figure 3 for response measurement procedure.

A: SPONTANEOUS REVERSAL MAGNITUDE

B: SPONTANEOUS REVERSAL FREQUENCY
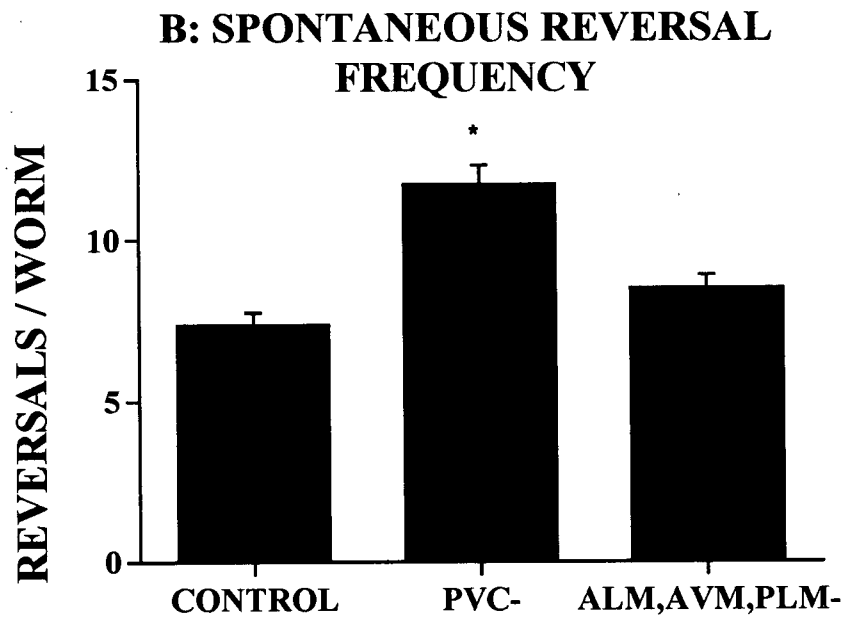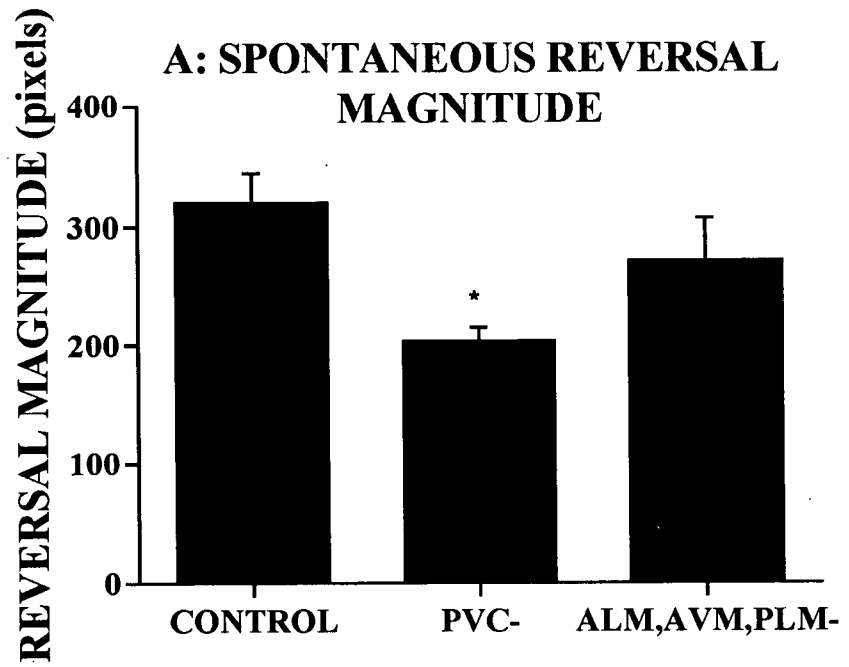
Experiment 5C

The observation that both the acceleration response—initiated by the posterior tail-touch receptors—and the reversal response—initiated by the anterior head-touch receptors—both habituated, and did so with distinct kinetics (Experiment 3; Wicks & Rankin, 1995b), suggested that the locus of habituation was either downstream of the mechanosensory neurons, and/or that it was distributed among the mechanosensory neurons; the locus was not specific to one mechanosensory channel or the other. Experiment 5B suggested that the mechanosensory neurons were not involved in either the generation or maintenance of spontaneous reversal activity.

To further restrict the possible loci of plasticity, the magnitude of spontaneous reversals that were not explicitly evoked by any stimulus both before and after habituation training were examined. It was reasoned that if the locus of habituation training was downstream of the sensory neurons (i.e., in the interneuron or motor neuron pools) then spontaneous reversals might also habituate as a consequence of training. If, however, the locus of habituation was modality or pathway specific, then it would be expected that the magnitude of spontaneous reversals would not change systematically as a consequence of habituation training.

Subjects.

A total of 92 hermaphroditic N_2 Caenorhabditis elegans were used.

Methods.

Subjects in this experiment were divided evenly into four groups. Two of the groups (EXP10, n = 23 and EXP60, n = 23) underwent habituation training, each receiving 40 stimuli, one group at a 10 s ISI, and the other at a 60 s ISI. The other two groups (CON10, n = 23 and CON60, n = 23) were treated the same as were the first two, but only received the first and last stimuli in the appropriate habituation series to assess responsiveness and to control for handling

effects and the effect of elapsed time since plating. Individual animals were gently transferred to individual testing plates with a platinum wire pick and were allowed to rest for 1 to 2 min. Then, 4 min of spontaneous locomotion was recorded, and the occurrence and magnitude of any spontaneous reversal was noted. Animals in each group then underwent the appropriate habituation training. Following habituation training, a further 4 min of spontaneous activity was recorded.

Scoring.

Spontaneous reversals were measured using the methods described for the measurement of tap-evoked reversals in the general methods.

Analysis.

An ANOVA was run on the magnitude post-training tap-evoked reversals observed in the four groups to assess responsiveness. Similarly, an ANOVA was run on the magnitude of spontaneous reversals observed in the four groups. In both cases, Fisher's PLSD were used to assess potential differences between groups. A Chi-squared analysis was used to assess differences in the frequency of spontaneous reversal emission between the groups.

Results.

The post-training tap-evoked reversal magnitude for animals trained at either a 10 s ISI or a 60 s ISI are shown in Figure 26A. These data are expressed as the percent of pretraining tap-evoked reversal magnitude. Animals in the CONTROL condition (10 s ISI, n = 23; 60 s ISI, n = 23) received sham habituation training, consisting of a tap at the onset and at the end of the training interval for that ISI. Animals in the HABITUATED condition (10 s ISI, n = 23; 60 s ISI, n = 23) received habituation training consisting of 40 tap stimuli. A two-factor ANOVA with factors for ISI (10 s vs. 60 s) and group (HABITUATED vs. CONTROL) was run on the magnitude of tap-evoked reversals. The only significant effect in the analysis was that of group,

Figure 26: The effect of habituation of the tap withdrawal response on spontaneous reversal

activity is shown. A) The tap withdrawal response magnitude after training in HABITUATED

and CONTROL animals is shown at two ISIs. The effects of training at two ISIs in the same two

groups on the magnitude (B) and frequency (C) of spontaneous reversal activity is also shown.

All three measures are expressed in terms of the percent pretraining levels. Error bars indicate SE.

See Figure 3 for response measurement procedure.

A: TAP WITHDRAWAL RESPONSE MAGNITUDE

B: SPONTANEOUS REVERSAL MAGNITUDE

□ CONTROL
■ HABITUATED

C: SPONTANEOUS REVERSAL FREQUENCY
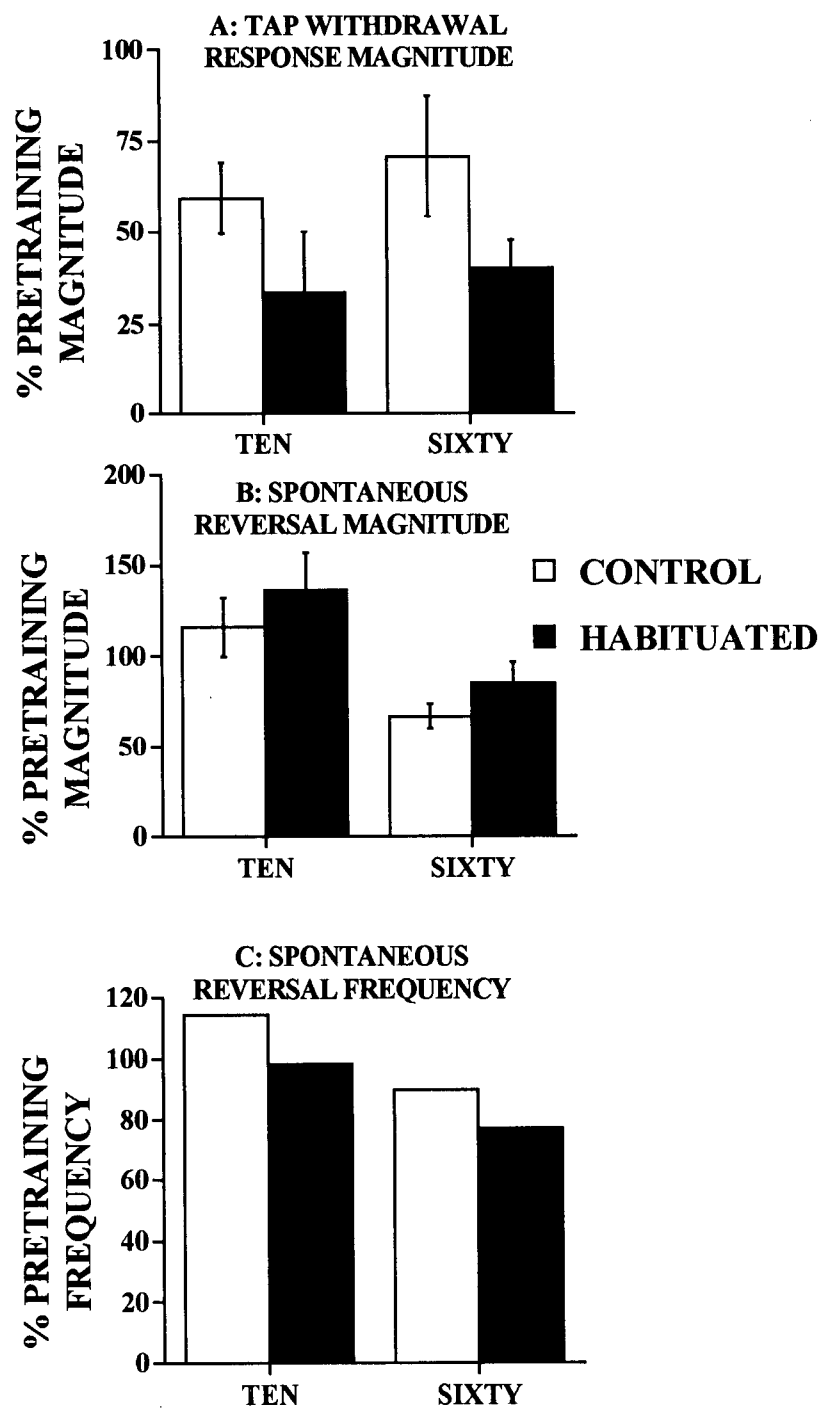
$F(1,74) = 4.42$, $p = 0.039$, suggesting that the tap withdrawal response in HABITUATED animals showed more decrement that did that of animals in the CONTROL group. There was no interaction between this effect and the ISI variable.

The averaged magnitude of spontaneous reversals for groups of HABITUATED and CONTROL animals trained at either the 10 s ISI or the 60 s ISI are shown for four minutes after habituation training in Figure 26B. These data are expressed as the percent of pretraining spontaneous reversal magnitude. A two-factor ANOVA with factors for ISI (10 s vs. 60 s) and group (HABITUATED vs. CONTROL) was run on the magnitude of spontaneous reversals. There was a significant ISI factor, $F(1,85) = 12.39$, $p < 0.0007$, which was indicative of a previously observed negative correlation between foraging-related activities, including spontaneous reversal frequency, and elapsed time since plating; immediately after platings worms begin active foraging (unpublished observations). Since it took longer to habituate animals in the 60 s ISI condition with 40 stimuli than it did for animals in the 10 s ISI condition, this effect was expected. More importantly, however, there was no significant main effect of habituation training, $F(1,85) = 1.85$, $p = 0.178$, nor was there any interaction between this variable and ISI, $F(1,85) = 0.008$, $p = 0.93$. Thus, the magnitude of spontaneous reversals did not habituate as a consequence of habituation of tap-evoked reversals.

The frequency of spontaneous reversals for animals in each of the four groups are shown in Figure 26C. A Chi-squared analysis (Siegel, 1956) was used to determine if there was a decrease in the frequency of emitted spontaneous reversals in habituated animals. Two-way contingency tables were constructed with factors for habituation (CONTROL vs. EXPERIMENTAL) and condition (BEFORE vs. AFTER). No differences were found at either a 10 s ISI, Chi-squared = 1.09, $p = 0.30$, or a 60 s ISI, Chi-squared = 1.41, $p = 0.24$, between the

Control and Experimental groups. That is, habituation at either a 10 s ISI or a 60 s ISI did not decrease the number of spontaneous reversals emitted by animals.

In summary, habituation training with tap stimuli decreased the magnitude of tap-evoked reversal responses. However, this manipulation had no effect on either the frequency or magnitude of spontaneously emitted reversals. Furthermore, the ISI at which animals are trained did not interact with any measure of spontaneous reversal activity.

Experiment 5D

That neither the frequency nor the magnitude of spontaneously emitted reversals decrement after habituation of the tap withdrawal reflex suggested that the circuitry involved in spontaneous reversal generation was not affected by this habituation. This experiment assessed whether another response that requires several of the tap circuit interneurons would be affected by habituation to tap. Several well characterized non-mechanical stimuli have been shown to be capable of modulating nematode locomotion, including volatile odorants (Bargmann et al., 1993; Colbert & Bargmann, 1995), chemosensory stimuli (Bargmann & Horvitz, 1991; Bargmann et al., 1990) and thermal stimuli (Hedgecock & Russell, 1975; Mori & Ohshima, 1995). Thermal stimuli were chosen for this experiment because it is possible to precisely control the temporal pattern of stimulus delivery. Furthermore, the neurons that transduce thermal stimuli have been characterized (Mori & Ohshima, 1995) and do not include the touch cells that transduce the mechanosensory stimuli used to evoke the tap withdrawal response (Chalfie et al., 1985; Wicks & Rankin, 1995a).

Subjects.

A total of 38 hermaphroditic N_2 Caenorhabditis elegans were used.

Methods.

Pairs of adult worms were isolated and plated onto individual foodless testing plates as described in the general methods section. After allowing subjects 1-2 min to recover from the transfer, the thermal responsiveness of each worm was assessed. This was done by bringing a heated probe into the path of a worm during forward locomotion, such that the probe was perpendicular to the body axis of the animal and contacted neither the worm nor the agar surface. The probe consisted of a scalpel blade heated in an alcohol flame until it glowed red. An attempt was made to allow the worm to swim into the proximity of the probe and hence set its own threshold for responding, although this was not always possible. Then worms were randomly assigned to either a CONTROL group or an EXPERIMENTAL group. Animals in the EXPERIMENTAL group (n = 19) received habituation training (40 stimuli at a 60 s ISI) whereas animals in the CONTROL group (n = 19) received just two tap stimuli, one at the beginning of, and one at the end of, a mock habituation run. The worms were then given to an assistant who randomly coded each animal. The magnitude of the reversal response to a second thermal probe was then measured. As this stimulus was hand-delivered, care was taken to ensure that the experimenter was naive to the experimental condition of any animal at this stage by recruiting a naive confederate to randomly key the animals. Thus, there was a delay of about 1 min between the end of the habituation run and the assessment of thermal responsiveness. Note that this delay may have been sufficient for some recovery from habituation to have occurred if animals were trained at a 10 s ISI (Rankin, unpublished observations). Thus, in this experiment animals were habituated at a long ISI (60 s) at which no recovery has been noted at intervals longer than this necessary delay.

<u>Scoring.</u>

The magnitude of reversals induced with a thermal probe were scored using the same methods as reversals induced with a tap stimulus.

<u>Analysis.</u>

Two dependent measures were assessed in this experiment. The response to both the first and last tap for animals in both the CONTROL and EXPERIMENTAL groups was assessed to determine the extent of habituation in these groups. The response to the last tap was expressed as a percentage of pre-training responding and analysed with a one sample t-test for each group. The second measure taken was the response magnitude of reversals induced by the thermal probe. Again, the post-training thermal response was expressed as a percentage of the pre-training response levels, and one-sample t-tests were used to determine if habituation training affected the animals responding to the thermal stimulus.

<u>Results.</u>

For each group, the magnitude of the response to the last tap was expressed in terms of a percentage of the magnitude of the first tap and is shown in Figure 27A. If there was no change in responsiveness as a consequence of habituation training, then the magnitude of the second tap should be 100% that of the first. A one-sample t-test was conducted on the mean response magnitude for both the CONTROL and EXPERIMENTAL groups. This analysis showed that both groups showed some decrement in responsiveness from the first to the last tap (EXPERIMENTAL, mean = 31.2, $\underline{t}$ = -5.96, df = 18, $\underline{p}$ < 0.0001; CONTROL, mean = 72.9, $\underline{t}$ = -2.64, df = 17, $\underline{p}$ = 0.017). A further investigation of these means demonstrated that the EXPERIMENTAL group had decremented more than the CONTROL group, $\underline{t}$ = -2.68, df = 35, $\underline{p}$ = 0.0056. It is unclear why the CONTROL group showed any decrement in this experiment; this observation is somewhat anomalous. However, it should be noted that a similar affect was

observed in Experiment 5C (see Figure 26A). Previously, Rankin (unpublished observations) has been unable to demonstrate response decrement with ISIs of 10 min or greater. Additionally, in studies of long-term habituation Beck and Rankin (1995) have shown that responding on day 2 was not different from responding on day 1. It is possible that the persistent appearance of this effect in these two experiments was due to small variances in the handling of the animals. However, since in both cases habituated animals show significantly more decrement than do non-habituated animals, the effect was not further explored.

The magnitude of the post-habituation response to the thermal probe, expressed in terms of a percentage of the pre-habituation response magnitude, is presented in Figure 27B. One sample t-tests confirm that neither the EXPERIMENTAL group (mean = 157.9, t = 1.46, df = 18, p = 0.16) nor the CONTROL group (mean = 96.3, t = -0.24, df = 18, p = 0.82) showed a significant change from prehabituation levels.

In summary, the magnitude of temperature-induced reversals did not change as a consequence of habituation training. This suggests that in addition to being upstream of the interneurons that control locomotion, the locus of habituation of the tap withdrawal reflex is pathway specific and limited to the mechanosensory modality.

Discussion of Experiments 5A-5D

In these experiments, the possible loci of change that might underlie TWR habituation were determined by studying the effects of repeated tap stimulation on other reversal behaviors. Reversals—either spontaneously emitted or elicited by a thermal stimulus—were measured before and after habituation of the tap withdrawal reflex. The tap-evoked reversal response showed a decrement in magnitude typical of habituation. However, neither spontaneously emitted reversals nor heat-evoked reversals showed any decrement following habituation of the TWR. As it was further shown that spontaneous reversal activity and reversal activity elicited

Figure 27: The effects of habituation of the tap withdrawal response magnitude and the

magnitude of reversals evoked with a thermal probe. Both measures are expressed in terms of

prehabituation levels of responding. See Figure 3 for response measurement procedure. Error bars

indicate SE.
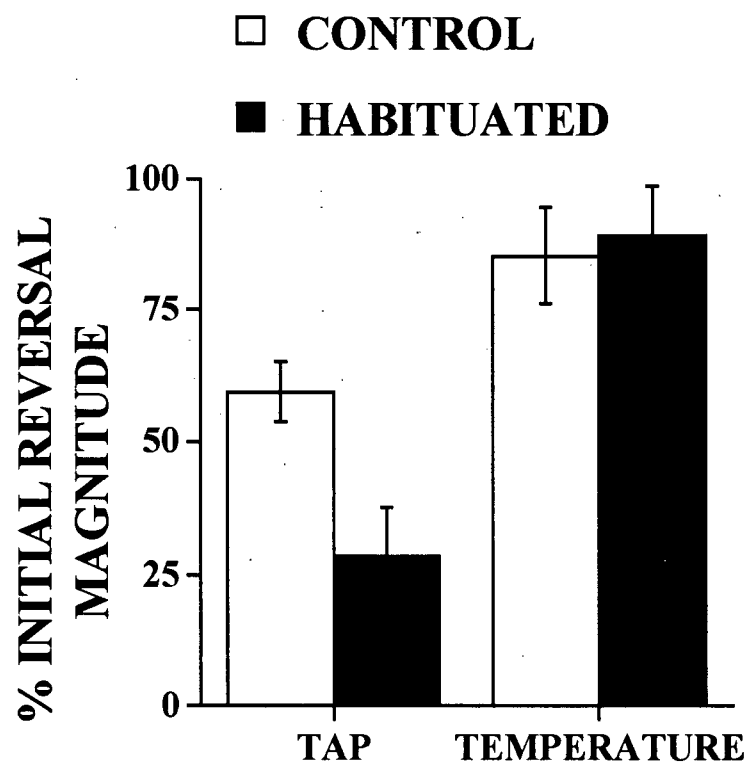
by the tap stimulus probably invoke the same neural circuit, these results suggest that the most likely loci of plasticity in the tap withdrawal circuit lie upstream of the neurons and musculature that control locomotion, and within a mechanosensory-specific pathway. Thus, the "final common pathway", as described by Sherrington (Sherrington, 1906), does not express changes induced by habituation of the tap withdrawal response.

The first experiment reported here attempted to establish evidence that the motor pattern that was invoked by tap stimulation was the same as that which was invoked by the production of a spontaneous reversal. Croll's (1975) analysis of reversal behavior was used to demonstrate that spontaneous and tap-induced reversals share a similar morphology, and can each be distinguished from another class of reversal responses: those induced by nose touch. All three behaviors utilize the same set of motor neurons for production, and as ablations of AVB or AVA either with a laser microbeam (Chalfie et al., 1985), or through ectopic expression of the toxic MEC-4 gene product (Maricq et al., 1995) result in uncoordinated worms that are incapable of coherent locomotion, all three behaviours likely activate these two command neurons as well. Thus differences in the three observed behaviours are likely due to differential modes of activation of this locomotion pattern generator. That is, distinct patterns of sensory input result in differences in the pattern of locomotion either elicited by a stimulus or spontaneously emitted. Although the nose touch and tap stimuli are mechanosensory in nature they are transduced by different neurons. Nose touch is transduced by the ASH neuron (Kaplan & Horvitz, 1993), and this neuron imparts mechanosensory information to the locomotion pattern generator via a glutamate receptor (GLR-1) that is expressed on the AVA, AVB and AVD interneurons (Hart et al., 1995; Maricq et al., 1995). Tap, on the other hand, is transduced by the touch cells (Chalfie & Sulston, 1981; Chalfie et al., 1985; Wicks & Rankin, 1995). Since the pattern of synaptic input to the locomotion CPG for each of these two behaviours is distinct, the pattern of behaviour

produced by each mechanosensory mode would also be expected to be distinct. In addition, it has been shown that a single anatomical pattern of synaptic input can have multiple modes of activity. For example, the ASH neuron not only transduces the nose-touch stimulus, but also transduces osmotic stimuli. The response to osmotic stimuli is unaffected by mutations in the *glr-1* gene that eliminate the worm's response to nose-touch (Hart et al., 1995; Maricq et al., 1995). Thus, patterns of synaptic input as well as receptor-transmitter interactions can both code for particular response strategies. Experiment 5A suggests that the motor patterns produced during a spontaneous reversal and produced by tap were the same.

Experiment 5B was conducted to determine the extent to which the neurons of the tap withdrawal circuit were involved in the production of spontaneous locomotion. The results confirmed that the touch cells did not play a part in either the initiation or the maintenance of spontaneous reversal activity under normal circumstances. Ablations of ALM, AVM and PLM resulted in animals that exhibited spontaneous reversals with normal frequency and magnitude. The PVC and AVD interneurons could serve exclusively as relays for mechanosensory input. Alternatively, these neurons might in addition participate in the production of locomotion more broadly. Since the ablation of the PVC interneuron affected both the frequency and magnitude of spontaneous backward locomotion, this neuron (and probably it's forward locomotion counterpart, AVD) must be considered part of the locomotion CPG. Although these results cannot rule out the possibility that a locus of change lies in the PVC and AVD cells (and the synapses formed by these neurons), both results reported in Experiment 5B, and the extensive reciprocal connectivity of these neurons within the locomotion pattern generator, suggest that this possibility is unlikely. Any change in synaptic efficacy in this feedback laden network would probably be reflected in the magnitude of spontaneous reversal activity. On the other

hand, the touch cells are exclusively presynaptic to the presumed CPG circuitry and thus remain strong candidates for possible loci of plasticity.

In Experiment 5C, it was shown that spontaneous reversal activity was not affected by habituation of the tap withdrawal response, despite the observations (from Experiments 1—2) that suggest that the two behaviors share substantial circuitry. The behavioral analysis of a spontaneous reflex magnitude during habituation has been used previously to confirm that the decrement of the defensive gill-withdrawal reflex in Aplysia was not due to fatigue of the gill musculature (Pinsker et al., 1970). In that study, Pinsker and colleagues demonstrated that habituation of the gill-withdrawal reflex did not result in a decrease in the magnitude of spontaneous contractions of the gill-mantle shelf.

In other organisms it has been possible to localize sites of habituation using an electrophysiological analogue of this approach. For example, quantal analysis was used by Castellucci and Kandel (1974) to establish a presynaptic locus of habituation of the Aplysia gill withdrawal reflex. This work was extended to an analysis of spontaneous miniature potential release in cultured cells that suggested that the presynaptic mechanism of synaptic depression was mediated by a functional uncoupling of excitation and secretion rather than transmitter depletion (Eliot, Kandel & Hawkins, 1994). These results were in accord with the results of Glanzman and Thompson (1980) who showed that the magnitude of spontaneous miniature potentials in the isolated frog spinal cord did not decrease as a consequence of habituation training of the lateral column-motoneuron monosynaptic pathway. Another example of this approach can be found in the analysis of habituation in the land slug Ariolimax. Chan and Moffet (1981) eliminated the neuromuscular junction as a potential locus of habituation of the tentacle withdrawal response by directly stimulating the L4 motoneuron while measuring muscle tension; no decrement in muscle tension was noted under these conditions.

Since electrophysiological analysis of neurons in situ in the worm is not yet practical, the simplicity of the worm nervous system was exploited to identify or exclude potential sites of neural change in C. elegans. The complete connectivity of each of the 302 neurons in the adult nervous system has been mapped (Hall & Russell, 1991; Ward et al., 1975; Ware et al., 1975; White et al., 1986). Previously, this has made it possible to describe the neural circuits that underlie a variety of behaviours (see Experiment 1). In this report, this simplicity was exploited to causally relate *changes* in behaviour to changes within the nervous system. The results of the experiments in this study suggest that the neural substrate of tap withdrawal response habituation is upstream of both the worm musculature and the neuronal pattern generator that controls locomotion.

It is also possible that the changes in the nervous system are extrinsic to the locomotion CPG, and are not necessarily in the mechanosensory pathway. That is, it is also possible that the changes associated with habituation of the tap withdrawal reflex are expressed as a change in state, rather than the tap-specific S-R system. Such a change might alter the responsiveness of an animal and impact only the magnitude of evoked responses, leaving spontaneous behaviour unmodulated. To assess this possibility, the animal's response evoked by a discrete non-mechanosensory stimulus (i.e., temperature), before and after habituation of the tap withdrawal reflex, was measured in Experiment 5D.

Temperature was chosen as a stimulus for several reasons. First, the neural circuit that transduces a thermal stimulus has been described (Mori & Ohshima, 1995) and is not the same as the circuit that transduces the tap stimulus. The specific neural pathways by which a thermal stimulus may control locomotion have not been explicitly described, although the RIM, AIY and AIZ interneurons are thought to play a key role in the integration of thermal stimuli (Mori & Ohshima, 1995). Second, since the thermal probe touched neither the worm, nor the substrate on

which the worm moved, it was possible to assure that the stimulus had no explicit mechanosensory dimension. This is true of a chemical stimulus, which might, for example, affect the hydration of the agar, and hence its biomechanical properties. Finally, it was possible to discretely control the rate and duration of stimulus presentation, and hence assure that stimulus properties unrelated to modality (e.g. duration) were similar to the tap stimulus. Habituation of the tap withdrawal reflex resulted in a decrement in the magnitude of tap-evoked reversals, but had no effect of the magnitude of temperature-evoked reversals. This suggests that the physiological correlates of habituation of the tap withdrawal reflex are mechanosensory pathway (i.e., S-R) specific and are not expressed as a decreased sensitivity to any eliciting stimulus (i.e., a change in state).

The results of these experiments suggest that the neural correlates of habituation of the tap withdrawal response are localized among the touch cells. Previously, it has been demonstrated that both the reversal component and the acceleration component of the tap withdrawal response habituate (Wicks & Rankin, in press-a). This, taken together with the results presented here, suggests that the loci of habituation are distributed among the touch neurons, rather than being specific to either the head-touch or tail-touch pathways. The observation that each component of the intact response habituates with distinct kinetics may reflect the fact that the patterns of synaptic input from the anterior and posterior mechanosensory circuitry onto the locomotion CPG are different. That is, distinct sets of post-synaptic partners, and different relative numbers of electrical and chemical connections from each of the anterior and posterior input to the tap withdrawal circuit might, in addition to coding for distinct responses to mechanosensory input, also allow these two pathways to respond differentially to repeated stimulation.

Knowledge of the complete neuronal connectivity (White et al., 1986) coupled with our circuit analysis of habituation (Experiment 3; Wicks & Rankin, in press-a), places us in a position to understand habituation in ways currently unavailable in other model systems. It is evident that the distinct nature of the tap-induced reversal and acceleration response habituation kinetics are a consequence of both the circuit as well as the cellular/molecular characteristics of the underlying neural substrate. In the current studies we have exploited this neural simplicity to identify potential sites of tap withdrawal response habituation. Knowledge of these sites will allow us to begin a molecular dissection of habituation using genetic techniques. These results demonstrate that to fully understand the cellular basis of habituation, a knowledge of the molecular mechanisms involved must be complemented by a detailed understanding of the circuit connectivity.

A Synthesis

The experiments reported here investigated the cellular substrate of a simple withdrawal response to a mechanosensory stimulus. The tap withdrawal response of C. elegans exhibits habituation; the simplicity of the nematode nervous system combined with the tractability of this organism in the fields of development and genetics urges the experimental analysis of this modulated behaviour. The objective of this research was to delineate the circuitry that maintains the withdrawal response. In addition, a number of functional properties of this circuitry were identified, both with respect to the production of the withdrawal response and with respect to the modulation of that response.

In Experiment 1, the sensory and interneurons that integrate mechanosensory information in C. elegans were identified. Specifically, a program of single-cell laser microsurgery was undertaken to ablate individual neurons. The impact of these lesions on the tap withdrawal response then was identified. The integration of the tap withdrawal response was contrasted with the integration of the animal's response to a light touch as described by Chalfie et al. (1985). The touch circuit and the tap withdrawal circuit can be distinguished on the basis of the complement of neurons that comprise each circuit. The sets of cells overlap, but are not identical. Specifically, the tap withdrawal circuit does not contain the LUA interneurons, but does contain two additional classes of neurons not found in the touch circuit: PVD and DVA.

Two properties of the stimulus (i.e., tap) were essential to the understanding of circuit functioning. First, the tap stimulus was graded and of repeatable intensity. The touch used by Chalfie, being hand delivered, was not of a quantifiable intensity. In contrast, the tap stimulus was delivered with an electromechanical relay and thus allowed fine control over intensity from trial to trial. This allowed the quantitative assessment of response magnitude, and thus the

relative effects of several ablations on a particular dependent variable (e.g. acceleration magnitude). Second, in contrast to the touch stimulus, the tap was a diffuse and non-directed stimulus. It activated both the head- and tail-touch pathways simultaneously. Thus, the tap withdrawal response was found not to be unitary, but rather was composed of two competing reflexive responses—forward locomotion in response to posterior mechanosensory stimulation and backward locomotion in response to anterior mechanosensory stimulation. Sherrington (1906) first recognised that a critical aspect of nervous system function was to produce adaptive behaviour even in the face of contradictory or ambiguous sensory input. The tap withdrawal reflex is one example of how this integration is accomplished in an intact system. On the basis of the data from Experiment 1, it was hypothesized that the chemical connections from the mechanosensory neurons (i.e., ALM, AVM and PLM) were acting to functionally inhibit or shunt the contrary response. That is, activation of ALM, for example, would initiate backward locomotion by exciting the appropriate interneurons via a gap junction, but would also actively inhibit the interneurons responsible for the induction and maintenance of forward locomotion via a set of chemical connections (see Figure 13).

An important assumption made in this work was that the effect on behaviour produced by the ablation of a cell was due to the loss of that cell's function directly on that behaviour. However, there are a number of caveats which need to be kept in mind when discussing the interpretation of ablation results. In general, any lesion may produce an effect if the lesioned area is permissive to a behaviour, even if the lesioned area is not actually contributing directly to that behaviour. This limitation of lesion studies also applies to the ablation of single neurons in a circuit. Also, when there are recurrent connections within a circuit, it can sometimes be difficult to interpret the consequences of the ablation of a neuron (although, as discussed below a computational modeling approach can alleviate this concern somewhat). The effects of an

ablation may be more accurately attributed to the alterations in complex interactions between circuit elements than to the loss of a specific role of the target cell. A further caveat of this work is that the ablations reported in these experiments were all done early in the development of C. elegans, and it is possible that the nervous system of these animals has compensated for the loss of these connections. Although this is an important consideration, it is clear that if there is compensation, it is not complete for many of the ablations reported in these experiments, since function was often profoundly modulated by ablation.

The first experiment suggested an informal model of how the tap withdrawal circuit integrated mechanosensory information. Experiment 2 formalized this model, and tested these predictions with respect to neuronal polarity using a simple dynamic network simulation of the tap withdrawal circuit. This instantiation of the model allowed the precise communication of how the circuit was thought to operate. In addition, because the formulation of the network simulation of the circuit forced explicit assumptions with respect to physiological variables, it allowed the formation of non-intuitive predictions. Specifically, by allowing the chemical synapse polarities of the circuit to vary, and by using behavioural criteria to optimize circuit output, it was possible to specify what connection polarities were correlated with good fits to the behaviour of real worms. The behavioural criteria used to fit the model data was the relative tap withdrawal response magnitude of worms that were distinguished by the ablation of identified neuron classes. This simulation predicted that neuronal polarities cluster according to anatomically defined classes that confirm and extend the informal models of Chalfie (1985) and Wicks and Rankin (1995a).

This novel experimental approach was based on the observation that behaviour undergoes "graceful degradation" as a consequence of lesions to the underlying neural substrates of that behaviour. That is, lesions do not completely disrupt the ability of a biological system to

respond adaptively to stimulation. Unlike an engineered physical system, biological systems respond commendably to lesion. The adaptive value of this ability is clear. Biological systems that are incapable of operation in the presence of trauma (which might be as innocuous as the effects of age or a transient disease process) would be at a selective disadvantage in a population, especially since such systems possess considerable regenerative capacity.

Once the tap withdrawal circuitry was identified, it was then possible to start exploring the cellular and molecular basis of simple forms of behavioural plasticity observed in the tap withdrawal response. In Experiment 3, it was demonstrated that cellular ablation of elements of the tap withdrawal circuit modulated habituation dynamics in predictable ways. These observations have implications for the identification of possible sites of plasticity in the circuitry which would facilitate a molecular/genetic analysis. If there was a single locus of plasticity downstream of both mechanosensory channels, the kinetics of habituation might be expected to be uniform. That each of the two reflexive components of the behaviour habituated with distinct kinetics suggested that each mechanosensory channel was modulated at a distinct locus. Furthermore, no single lesion abolished the animal's ability to habituate, suggesting that even the modulation of behaviour is also subject to the rule of graceful degradation. These observations are consistent with the concept that distributed changes in a nervous system underlie learning (Falk et al., 1993; Zecevic et al., 1989).

The tap withdrawal reflex is an adaptive behaviour. The worm is a non-parasitic free-living nematode; its natural ecology consists of damp soil. The mechanosensory withdrawal reflex probably developed to allow the animal to escape from a directional stimulus; the worm does have a simple yet defined mechanosensory somatotopy. However, the worm is very small relative to many of the potential sources of mechanosensory noise in its environment, which might include vibrations produced by the footfalls of animals, rainfall or falling debris that are

mass-transmitted through the soil. Under normal conditions the animal may therefor be responding to relatively non-directional stimuli such as the tap. The shift in the frequency of response type and the decrease in reversal magnitude observed during habituation could be interpreted as an alteration in response strategy with ethological consequences for the animal. A foraging worm which receives a single or very few vibrations might be well advised to first reverse and then resume forward motion in a new direction and thus avoid an area which is unsuitable for habituation. However, an animal which is being chronically stimulated may find itself in the midst of such an area, and would be better off navigating the fastest course to the boundary, an effort which is best served by accelerating in the same direction to each stimulus, rather than repeatedly reversing.

Frequency dependent recovery from habituation is one of the defining characteristics of this form of learning (Thompson & Spencer, 1966). Experiment 4 was conducted to determine whether the kinetics of recovery from habituation were truly dependent on the frequency of stimulation during training as suggested by the literature, or whether the kinetics of recovery were rather dependent on the behavioural consequences of training at a given frequency. The results of this experiment suggested that not only were the kinetics of recovery ISI-dependent, but that this process was distinct from the ISI-dependency of habituation kinetics. These results can be used to experimentally isolate these two process during mutant screens to identify mutations in which one process or the other is specifically affected.

Several lines of theoretical and empirical evidence have suggested multiple processes are recruited during habituation. For example, Engel and Wu (1996) have shown that cAMP-pathway mutants preferentially disrupt habituation kinetics of the long-latency giant fiber response in Drosophila without strongly affecting the kinetics of recovery from habituation. Byrne (1982) has similarly concluded that, at the sensory neuron synapse mediating the Aplysia

gill withdrawal reflex, multiple functions underlie recovery from synaptic depression. The dual-process theory of habituation (Groves & Thompson, 1970) suggests that a facilitating component and a decrementing component underlie habituation of a given response. These process are thought to be physiologically distinct; the decrementing process is thought to be S-R specific, while the facilitating process is thought to be a state variable. There is no reason to believe that these process then must necessarily recover at similar rates. Indeed, results presented in Experiment 3 suggest that a facilatatory subcomponent of the response curve is preferentially recruited at short ISIs, which confirms Byrne's (1982) hypothesis that recovery at short ISIs is a nonlinear summation of two or more processes, one of which is a facilitation—perhaps due to calcium loading—evoked by high frequency stimulation.

Finally, in Experiment 5, several questions concerning the locus of memory storage in this system were explored. The approach used in this experiment was to identify circuitry involved in reversal behaviours that were not evoked by mechanosensory stimulation and determine whether these alternate behaviours were affected by habituation of the tap withdrawal response. This analysis is an extension of the concept of the "final common pathway" (Sherrington, 1906). Several behaviours involve locomotion via well-defined circuitry. As habituation of the tap withdrawal response did not modulate these behaviours it was inferred that the locus of change must be upstream of this circuitry. A similar logic was used by Engel and Wu (1996) in the localization of giant fiber escape response habituation in <u>Drosophila</u>.

The results of this analysis suggest that the most likely loci of habituation are the mechanosensory neurons (or their synaptic terminals). These cells express a well defined genetic program (Hamelin, et. al., 1992; Mitani et al., 1993; Savage et al., 1989) that has been exploited to efficiently control gene expression exclusively in the touch mechanosensory neurons (Fire, 1986; Fire, Harrison & Dixon, 1990). Thus, in the future, reverse genetic technology in the worm

(Plasterk, 1992; Zwaal et al., 1993) can be utilized to specifically examine putative molecular underpinnings of habituation in C. elegans. In addition, other cells in the tap withdrawal circuit are also genetically characterizable; gene expression in these cells may be manipulated either directly (Hart, Sims & Kaplan, 1995; Maricq, Peckol, Driscoll & Bargmann, 1995) or indirectly with a laser microbeam (Stringham & Candido, 1993).

Although homosynaptic depression (Castellucci et al., 1970; Castellucci & Kandel, 1974; Kupfermann et al., 1970) has been advanced as a well-developed model of the physiological correlates of habituation, a number of viable alternative mechanisms have been proposed (Fischer & Carew, 1993; Krasne & Teshiba, 1995). Since these experiments suggest that changes in the mechanosensory neurons constitute the substrate of plasticity of each subcomponent of the withdrawal response, and since these neurons are further thought to possess an inhibitory synaptic phenotype, the habituation of this response, if it involves the chemical synaptic terminals of these cells, must be instantiated as a modulation of inhibitory neurotransmission. The Aplysia model of homosynaptic depression is a downregulation of an excitatory response, and thus is not a good candidate mechanism for the modulation of C. elegans behaviour. The data, at first glance appear to be compatible with the theory proposed by Ratner (1970) regarding habituation, in which response decrement is due to the appearance and eventual predomination of alternative behaviours. The initial reversal reflex of the tap withdrawal response decrements because of the appearance of the antagonistic acceleration reflex. This mechanism does appear to explain some fraction of the decrement in responding observed, especially at short ISIs, however, it can not explain why each subcomponent of the behaviour decrements independently.

Two further alternatives exist that must be considered. First, several roles for inhibition have been proposed to account for changes in behaviour. Krasne (1995) has suggested that the slow development of active inhibition of the reflex arc from encephalic centers underlies a

significant fraction of the habituation of the crayfish tailflip response. Although the definition of "higher" or more central fractions of a nervous system are somewhat problematic in an organism as simple as the nematode, it is still possible that such a mechanism might account for even pathway-specific response decrement in the worm. The action of a neurohormone or systemically-released peptide might, for example, act directly on the sensory neurons and downregulate their activity. Fischer and Carew (1993) have also proposed a possible role for inhibition as a mechanism of habituation. They propose that activity-dependent potentiation of recurrent inhibition between the L30 and L29 interneurons of the Aplysia abdominal ganglia may play a significant role in habituation of the gill withdrawal reflex. As outlined in Experiment 2, the tap withdrawal circuit contains the circuit configuration necessary to express this form of plasticity, so this remains a possibility. However, this possibility suggests a reinterpretation of the results of Experiment 3. It was asserted that, since the kinetics of habituation of the head- and tail-touch channels were distinct, the locus of habituation of the intact response was probably upstream of the interneuronal pool. If activity-dependent potentiation of recurrent inhibition is accepted as a mechanism of habituation in this system, then it can only explain the results of Experiment 3 by suggesting that each channel habituates because of a change at a "central" site, and that the channel-specific kinetics are due to differential recruitment of the affected synapse by each channel.

A second physiological alternative to Ratner's (1970) molar theory of habituation is that the modulation of behaviour is due to the change in the efficacy of excitatory electrical synapses between the sensory neurons and interneurons of the tap withdrawal circuit. Although the roles that the modulation of electrical transmission may play in plasticity have not been well developed, electrotonic coupling is common in nervous systems (Bennett, 1972), and can be modulated under a variety of conditions. In the vertebrate retina, for example, gap-junction

conductance can be decreased via a cAMP-dependent mechanism (Piccolino, Neyton & Gershenfeld, 1984). Ample evidence supports a major role for cAMP-dependent pathways in various forms of activity-dependent plasticity (Byrne, Zwartjes, Homayouni, Critz & Eskin, 1993; Dash, Hochner & Kandel, 1990; Eskin, Garcia & Byrne, 1989; Kaang, Kandel & Grant, 1993; Martin & Koshland, 1992; Yin, Del Vecchio, Zhou & Tully, 1995; Yin. et al., 1995), so such a mechanism of habituation is a plausible one.

With the rapid sequencing of the C. elegans genome (Waterson et al., 1992) the stage is set to identify gene products involved in the experience dependent modulation of behaviour in this system utilizing both classical and reverse genetic approaches. This, in combination with the type of studies presented here, makes C. elegans a powerful tool for the understanding of the processes underlying learning. The strength of the nematode as a model neurobiological system is that it is not necessary to study a particular component of the system in isolation; with only 302 neurons to account for its entire behavioural repertoire, the possibility exists of describing the roles of every neuron in the organism in every behaviour that the animal exhibits. Ultimately, the power of nematode genetics will allow us to complete this analysis of habituation with a description of the molecular mechanisms underlying the observed changes in behaviour. This description of the relationship between the behaviour and the circuitry that mediates that behaviour is a requisite first step in such an analysis.

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Appendix 1

<u>The Neuron</u>

The neurons of <u>C. elegans</u> have simple morphologies which are preserved across individuals. Many neurons consist of a single unbranched process, and few have more than two branches (Chalfie & White, 1988). Electrophysiology on <u>C. elegans</u> cells is still in its infancy (although see Avery et al., 1995; Raizen & Avery, 1994); little is known about the membrane characteristics of its neurons. However, electrophysiology has been done on <u>Ascaris</u>, a larger nematode related to <u>C. elegans</u> (Davis & Stretton, 1989a; Davis & Stretton, 1989b). Its dorsal and ventral nerve cords have been reconstructed and show considerable similarity to those of <u>C. elegans</u>, and homologues of <u>C. elegans</u> motor neurons have been found in <u>Ascaris</u> (Chalfie & White, 1988; Stretton et al., 1992). For this model, electrophysiological data from <u>Ascaris</u> was used to determine model parameters.

Evidence from <u>Ascaris</u> suggests that signal propagation in <u>C. elegans</u> neurons is likely accomplished electrotonically, without classical all-or-none action potentials. Intracellular recordings of <u>Ascaris</u> motorneurons and interneurons show no evidence of action potentials, nor has it been possible to evoke them (Davis & Stretton, 1989a). Membrane resistivity in <u>Ascaris</u> is unusually high ($60 - 300$ kΩcm^2), and is within the range that would permit signal propagation without action potentials. Niebur and Erdös (1993) have used <u>Ascaris</u> data to do detailed computational studies of the electrotonic characteristics of <u>C. elegans</u> neurons and have shown that the integration of <u>C. elegans</u> locomotion can be accounted for by purely electrotonic signals.

Davis and Stretton (1989a) have measured specific membrane resistances $\hat{R}_m$ and intracellular resistivity $\hat{R}_i$ in <u>Ascaris</u>. In four motorneurons, $\hat{R}_m$ varied from $89 - 251$ kΩcm^2. and $\hat{R}_i$ from $79 - 314$ Ωcm. We assumed that membrane properties in <u>C. elegans</u> were similar, and used an average of the four measurements: $\hat{R}_i = 180$ Ωcm, and $\hat{R}_m = 150$ kΩcm^2. We

assumed a specific membrane capacitance of 1 μF/cm², a standard value for a lipid bilayer (Rall, 1989). These membrane properties were adapted to C. elegans anatomy by using the surface area of each cell (see Table 3). Each neuron's branching morphology is given in Wood (1988b) and White et al. (1986). This, together with measurements of electron micrographs in White et al. (1986) and White et al. (1976) were used to determine average process lengths and diameters. Diameters varied from 0.2 to 1.0 μm, and an average value of d = 0.5 μm was used.

Process lengths were taken from diagrams in Wood (1988b), assuming a standard worm length of 1 mm. Soma diameters were taken from camera lucida drawings in Wood (1988b). Soma diameters varied from 2 μm to 10 μm and we used an average diameter of 5 μm. From these data, a total membrane surface area for each cell was computed and the resulting total membrane capacitance and resistance for the entire cell was derived (see Table 3). For simplicity it was assumed that cells were isopotential. Since the length constant (Rall, 1989),

$$\lambda = \frac{1}{2}\sqrt{\frac{d\hat{R}_m}{\hat{R}_i}}, \tag{4}$$

was generally longer than the process (data not shown), this was reasonable (also see Table 3). Thus a neuron's membrane potential, V, was governed by the usual single-compartment membrane equation (Segev, Fleshman & Burke, 1989),

$$C_m \frac{dV}{dt} = \frac{1}{R_m}(V_{leak} - V) + \Sigma I_{SYN} + I_{EXT}, \tag{5}$$

where C_m is the total membrane capacitance for the cell, R_m is the total membrane leakage resistance for the cell, V_{leak} is the leakage potential of the cell, I_{SYN} is the current due to a

synaptic input, and I_{EXT} is any injected current. A value of -35 mV was used for the leakage

potential in these cells (R.E. Davis, personal communication, May 1995).

Gap Junctions

The anatomical reconstruction of the nematode nervous system allowed the identification

of both electrical and chemical synapses (White et al., 1986). Gap junctions were modeled as

ohmic resistances where current flowing into cell i from cell j was given by,

$$\hat{I}_{ij} = \hat{g}_{ij}(V_j - V_i), \tag{6}$$

where $\hat{g}_{ij}$ was the conductance of the gap junction. Niebur (1988) used a standard conductance

per unit area value of 1 S/cm^2 (Bennett, 1972) and used unpublished micrographs to determine

the area of each gap junction. He reported that gap junctions ranged from 0.2 - 2 μm in length and

were 0.5 μm in width (Niebur, 1988). In our model, a standard gap junction length of 1 μm was

assumed, with a resulting conductance of 5 nS for all gap junctions. In some experiments, this

value was increased or decreased by a factor of ten to test the sensitivity of the model's

predictions to the precise value of the conductance used.

Synapses

Synaptic classes consisted of a number of individual synaptic contacts. The number of

contacts in each class was extracted from an anatomical database of C. elegans synaptic

connectivity (Achacoso & Yamamoto, 1992). The identification of chemical synapses from the

anatomical reconstruction was done by identifying presynaptic specializations, and inferring

postsynaptic partners on the basis of proximity; no postsynaptic specializations were evident in

the electron microscope reconstructions (White et al., 1986). Any error associated with this

technique would tend to overestimate the number of chemical synapses in the organism, however,

and any spurious synaptic classes would not be duplicated with high frequency. Therefore, such synapses, although present in the model would not be assigned a large synaptic weight and would thus not have a large impact on the response of the circuit to stimulation. Each modeled synapses represented a class of synaptic contacts with total synaptic conductance—the "weight"—given by the product of the number of individual contacts within the class and the individual synaptic conductance.

The synapse model used was based on the graded synapse model used by Lockery and Sejnowski (1992) in the leech local bending circuit. However, it was extended it to explicitly include the synaptic reversal potential as well as the conductance. Postsynaptic current was due to gated channels in the postsynaptic membrane with inward current given by,

$$I = g(t)(E_{SYN} - V_{POST}), \tag{7}$$

where $g(t)$ was the synaptic conductance of the postsynaptic membrane which was gated according to presynaptic potential, and E_{SYN} was the reversal potential for the synaptic conductance, which was assumed to be constant. For excitatory synapses, a reversal potential of 0 mV was used and for inhibitory synapses -45 mV was used (R.E. Davis, personal communication, May 1995). For simplicity, it was assumed that all synapses made by a single cell possessed the same reversal potential. This amounts to assuming that all synapses made by a given presynaptic cell were of the same polarity and class. Computationally, this reduced the number of optimized parameters—each of which possessed two possible values—to the number of neurons in the modeled circuit. It was further assumed that all modeled synapses functioned as fast ligand-gated channels. It was possible that some anatomically defined synapses were modulatory and acted through slow second messenger systems, or that synaptic function was

altered by the milieu interieur (Harris-Warrick, Nagy & Nusbaum, 1992), however, we assumed

that these modulatory effects did not affect a single tap withdrawal response.

In the leech local bending reflex, Lockery and Sejnowski (1992) observed multiple time

courses in some of the postsynaptic responses and to model this, they used a slow (10 ms) and a

fast (1500 ms) conductance, each governed by its own first order equation. Preliminary versions

of this model used a fast (10 msec) synaptic time constant, but no significant differences were

noted in the results of circuits containing these synapses and simulations which used an

instantaneous synapse. We therefore used a synaptic conductance that depended only on the

presynaptic membrane potential,

$$g(t) = g_\infty(V_{PRE}). \tag{8}$$

where g_∞ represents the steady-state conductance in response to a presynaptic voltage.

Synaptic Activation

No direct recordings have yet been made in C. elegans to determine properties of synaptic

activation. In recordings made from Ascaris commissural motorneurons, Davis and Stretton

(1989b) demonstrated that synaptic transmission is graded and transmitter is released tonically

between both excitatory and inhibitory motorneurons and postsynaptic muscle and

motorneurons. They found that changes in postsynaptic potential were related to presynaptic

depolarizing current by a sigmoidally shaped curve and that the presynaptic resting potential lies

approximately in the middle of the voltage-sensitive range of synaptic transmission.

Dynamic network simulations based on graded synaptic transmission have previously

been described (DeSchutter, Angstadt & Calabrese, 1993; Lockery & Sejnowski, 1992). We

assumed that synaptic activation and transmission in C. elegans was similar to Ascaris, namely

that it was graded and sigmoidally shaped with presynaptic potential, and was tonically active

with the equilibrium potential in the middle of the voltage sensitive range. Accordingly, we used a

symmetric sigmoidal function to model the steady-state postsynaptic membrane conductance,

$$g_\infty(V_{PRE}) = \frac{\overline{g}}{1 + e^{K\left(\frac{V_{PRE} - V_{EQ}}{V_{RANGE}}\right)}},$$ (9)

where $\overline{g}$ was the maximal postsynaptic membrane conductance for the synapse, V_{EQ} was the

presynaptic equilibrium potential at the middle of the voltage sensitive range and V_{RANGE} was the

presynaptic voltage range over which the synapse activated. We used a value of

$$K = 2\ln\left(\frac{0.1}{0.9}\right) = -4.3944,$$ (10)

so that the conductance changed from 10% to 90% of its maximal value over a presynaptic

voltage range of V_{RANGE}. Note that because synapses were tonically active, a cell's equilibrium

potential was not defined solely by its resting membrane potential, but rather was determined

from the fixed point of the entire system of equations governing the circuit. This was computed

prior to each run in the following way.

Equilibrium Potential

The assumption that tonically active synapses were active in the middle of their voltage

sensitive range at the equilibrium potential implied that the postsynaptic conductance g(t) was

one-half its maximal value $\overline{g}$ when the circuit was at equilibrium. Let V_i denote the membrane

potential for neuron i and similarly for other quantities pertaining to neuron i. Let I_{ij} denote the

synaptic current flowing into neuron i from neuron j across a single synapse and let ω_{ij} denote the

total number of synaptic connections from neuron j to neuron i. Similarly, let $\hat{I}_{ij}$ denote current

flow across a single gap junction in the direction from neuron j to neuron i and let $\hat{\omega}_{ij}$ denote the

total number of gap junctions between neuron j and neuron i . Finally, let I_i denote the external

current flow into cell i (caused by either sensory stimulation or external current injection). Then,

the entire system is given by:

$$R_{m_i} C_{m_i} \frac{dV_i}{dt} = V_{LEAK_i} - V_i + R_{m_i} \sum_{j=1}^{N} \left(I_{ij} + \hat{I}_{ij} \right) + I_i, \tag{11}$$

$$\hat{I}_{ij} = \hat{\omega}_{ij} \hat{g}_{ij} \left(V_j - V_i \right), \tag{12}$$

$$I_{ij} = \omega_{ij} g_{ij} \left(E_{ij} - V_i \right), \tag{13}$$

$$\frac{dg_{ij}}{dt} = \frac{g_{\infty_{ij}}\left(V_j\right) - g_{ij}}{\tau_{ij}}, \tag{14}$$

$$g_{\infty_{ij}}\left(V_j\right) = \frac{\overline{g}_{ij}}{1 + e^{K_{ij}\left(\frac{V_j - V_{EQ_j}}{V_{RANGE_{ij}}}\right)}}, \tag{15}$$

where N is the number of neurons in the circuit, and τ_{ij} is the synaptic time constant.

At equilibrium, $V_i = V_{EQ_i}$, and $\frac{dV_i}{dt}$, $\frac{dg_{ij}}{dt}$ and I_i are zero. Synaptic conductances are tonic

and at their half-activation at equilibrium, so that,

$$g_{\infty_{ij}}\left(V_{EQ_j}\right) = \frac{\bar{g}_{ij}}{2}. \tag{16}$$

After algebraic manipulation, this yields a system of linear equations which can be solved using standard Gaussian elimination to find V_{EQ_i} (Press et al., 1989 for example):

$$\mathbf{V}_{EQ} = \mathbf{A}^{-1}\mathbf{b}, \tag{17}$$

where A_{ij} is the ith row and jth column of matrix $\mathbf{A}$ and is given by

$$A_{ij} = -R_{m_i}\hat{\omega}_{ij}\hat{g}_{ij}, \qquad i \neq j, \tag{18}$$

$$A_{ii} = 1 + R_{m_i}\sum_{j=1}^{N}\left(\hat{\omega}_{ij}\hat{g}_{ij} + \omega_{ij}\bar{g}_{ij}/2\right), \tag{19}$$

and $\mathbf{b}$ is a vector which is given by

$$b_i = V_{LEAK_i} + R_{m_i}\sum_{j=1}^{N}E_{ij}\omega_{ij}\bar{g}_{ij}/2. \tag{20}$$

The computed equilibrium potential of cells varied within a physiologically realistic range from -47 mV to 0 mV, with a mean of -24 mV and a standard deviation of 13 mV. This did not vary appreciably from cell to cell, but depended rather on the circuit's polarity configuration and ablation condition. As an aside, the system (equation 17) can also be inverted to explicitly give V_{LEAK} in terms of V_{EQ_i}:

$$V_{LEAK_i} = V_{EQ_i} - R_{m_i} \sum_{j=1}^{N} \omega_{ij} \bar{g}_{ij} / 2\left(E_{ij} - V_{EQ_i}\right) - R_{m_i} \sum_{j=1}^{N} \hat{\omega}_{ij} \hat{g}_{ij} \left(V_{EQ_j} - V_{EQi}\right). \quad (21)$$

Synaptic Parameters

To determine values for V_{RANGE} and $\bar{g}$, the synapse model was fitted to published

measurements (Davis & Stretton, 1989b) of Ascaris muscle cell postsynaptic response to

presynaptic current injection as detailed below. Thus, values for V_{RANGE} and $\bar{g}$ for particular

Ascaris synapses were obtained. We assumed that C. elegans synapses activated over voltage

ranges similar to Ascaris synapses. However, the maximal synaptic conductance, $\bar{g}$, needed to be

adapted to C. elegans. We assumed that $\bar{g}$ represented the product of a synaptic conductance per

unit area and a synaptic area. In the case of a synapse mediated by a single population of ion

channels, $\bar{g}$ would be equivalent to the single-channel conductance of an open channel times the

total number of channels. To adapt the $\bar{g}$ value from Ascaris to C. elegans we assumed that C.

elegans synapses had similar unit-area conductances and accordingly scaled the Ascaris $\bar{g}$ by a

factor to account for the presumed difference in synaptic areas. We assumed that the total

synaptic area between two cells was proportional to the length of the process, which we

estimated by the ratio of body lengths—roughly 1/250. As this represents only a gross

approximation, the value of $\bar{g}$ used in these studies was varied over three orders of magnitude in

different experiments (see Results).

Modeling Ascaris Monosynaptic Responses

Data from Davis and Stretton (1989b Figures 13 and 14) show the postsynaptic

response of an Ascaris dorsal muscle cell (DM) to current injected into a presynaptic excitatory

motorneuron, DE1, and the response of a ventral muscle cell (VM) to current injected into a

presynaptic inhibitory motorneuron, VI. Both of these response profiles were sigmoidal in shape, were centered approximately at the resting potential, and had asymptotic saturated postsynaptic responses at the extremes of positive and negative presynaptic current injection. Therefore, the sigmoidal tonic response model presented here was well suited to fitting these synaptic responses.

Davis and Stretton (1989a; 1989b) placed a recording electrode in a muscle cell within the output zone of the motorneuron, and an injecting electrode at the ventral end of a commissural process leading to the synapse. The measured input resistance of the motorneuron was used to obtain the resulting membrane potential at the point of current injection, and an infinite cable model was used to determine the membrane potential at the presynaptic site. Since the recordings that were used to determine input resistance were made at the same ventral end of the commissure as was injected for the synaptic response measurements (R.E. Davis, personal communication, May 1995), and since the input resistance was approximately constant over the relevant range of injected current (Davis & Stretton, 1989a), it is possible to directly use the measured input resistance to determine the membrane potential at the point of current injection.

Davis and Stretton (1989a) determined the motorneuron cable properties by fitting their measurements along the commissure to an infinite cable model (Rall, 1989), and found that the length constant was unusually high ($\lambda \approx 8$ mm)—roughly the same magnitude as the length of the process. With such a large length constant, it is possible that the cable's branching morphology and sealed ends played a significant role in determining these cable properties (Rall, 1977) suggesting that a sealed end cable model might be more appropriate. However, for consistency we used an infinite cable model with cable constants as determined by Davis and Stretton (1989a), in order to reproduce their measured voltage response along the commissure.

Specifically, the presynaptic depolarization in response to an injected current is given by

$$\Delta V_{PRE} = I_{INJ} R_{PRE} e^{-L/\lambda}, \tag{22}$$

where L is the distance from the point of current injection to the synapse, and R_{PRE} is the input resistance at the point of injection. For the DE1-DM synapse, the distance, L, was 5—8 mm; for the VI-VM synapse, it was 0.5—2.5 mm (R.E. Davis, personal communication, May 1995), and we used the mean of 7 mm and 1 mm respectively. The input resistances for the DE1 and VI motorneurons were reported to be 6 MΩ and 17 MΩ respectively (Davis & Stretton, 1989a).

According to the sigmoidal tonic response model, the steady-state plateau response of the postsynaptic muscle is given by:

$$V_{POST} = V_{LEAK} + R_{POST} n g_\infty (\Delta V_{PRE})(E_{SYN} - V_{POST}), \tag{23}$$

where V_{LEAK} and E_{SYN} pertain to the postsynaptic muscle cell, R_{POST} is the postsynaptic cell's input resistance, $g_\infty(V_{PRE})$ is the steady-state synaptic conductance, and n is the total number of synapses between the motorneuron and the muscle cell. The input resistance of ventral and dorsal muscle cells was measured to be 0.18—0.50 MΩ, with a mean of 0.3 MΩ (R.E. Davis, personal communication, May 1995). Values of V_{LEAK} = -35 mV and E_{SYN} = 0 mV for excitatory and E_{SYN} = -45 mV for inhibitory reversal potentials were used (R.E. Davis, personal communication, May 1995). A light microscope study of dye-injected muscle cells in Ascaris suggested that the DE1 motorneurons make roughly 5—10 synapses to each dorsal muscle cell and the VI motorneurons make roughly 8—16 synapses to each ventral muscle cell (J. Donmoyer, personal communication, May 1995). Values from the high end of these ranges were adopted for n in equation (23) because muscle cells form gap junctions with each other (J.

Donmoyer, personal communication, May 1995), and thus it was possible that synapses from neighboring muscle cells played a role in determining a muscle cell's postsynaptic potential.

Equation (23) can be rearranged to yield values of V_{POST} explicitly in terms of V_{PRE}:

$$V_{POST} = \frac{V_{LEAK} + E_{SYN} R_{POST} n g_\infty(\Delta V_{PRE})}{1 + R_{POST} n g_\infty(\Delta V_{PRE})}, \tag{24}$$

where

$$g_\infty(\Delta V_{PRE}) = \frac{\bar{g}}{1 + e^{\kappa \frac{\Delta V_{PRE}}{V_{RANGE}}}}. \tag{25}$$

The change in postsynaptic potential was therefore given by

$$\Delta V_{POST} = V_{POST} - V_{EQ}, \tag{26}$$

where V_{EQ} is the equilibrium potential achieved by the postsynaptic cell under unstimulated in-circuit tonic synaptic input (see Equations (17)—(21)), and is given by

$$V_{EQ} = \frac{V_{LEAK} + E_{SYN} R_{POST} n \bar{g}/2}{1 + R_{POST} n \bar{g}/2}. \tag{27}$$

Equations (22) and (24)—(27) define a non-linear function for postsynaptic membrane potential in terms of presynaptic injected current and contain two unknown parameters, $\bar{g}$ and V_{RANGE}.

Levenberg-Marquardt's method (Press et al., 1989) was used to fit this function to the data from Davis and Stretton (Davis & Stretton, 1989b).

We obtained good results to the fit for the inhibitory VI-VM synapse. This fit included the reversal potential, E_{SYN}, as a fit parameter; this improved the fit substantially without significantly changing the reversal potential (-48 mV as opposed to -45 mV). The results of this fitting procedure yielded values of $\bar{g}$ = 150 nS, V_{RANGE} = 52 mV, E_{SYN} = -48 mV and were stable under various initial conditions. The DE1-DM fit was less precise since the steady-state conductance was not precisely a symmetric sigmoid. We therefore manually explored a number of parameter values and attempted to reproduce the approximate range and amplitude of activation. For this we obtained values of V_{RANGE} = 20 mV and $\bar{g}$ = 90 nS.

To adapt these values to C. elegans, an average of the two V_{RANGE} values was used to estimate the activation range (-35 mV), and the $\bar{g}$ from the VI-VM synapse fit was scaled by the 1/250 ratio of body lengths to yield a maximal conductance of 0.6 nS for an individual C. elegans synapse.