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Mechanisms of behavioral choice in the nervous system of the medicinal leech

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences

by

Quentin Gaudry

Committee in charge:

Professor William B. Kristan Jr, Chair
Professor Timothy Q. Gentner
Professor Jeffry S. Isaacson
Professor Sascha du Lac
Professor Massimo Scanziani

2008

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The Dissertation of Quentin Gaudry is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008

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Acknowledgments

I am eternally grateful to have had the opportunity to work with Dr. Bill Kristan. Bill is both a model scientist and mentor. Bill's experience and expertise have made my time at UCSD a true pleasure. Bill allowed me the freedom to explore my own scientific curiosities yet also taught me to focus on the goals and experiments at hand. Bill is a wealth of knowledge, and I appreciate all of the effort he has made ensuring my success in neuroscience. Bill has also made sure my scientific education extended beyond his lab at UCSD. I am thankful for the diversity of conferences and meetings he encouraged me to attend. I am also thankful to Bill for organizing a trip to Lake Itasca and providing me the opportunity to study leeches in their natural habitat. Bill is a strong advocate for teaching and education. I am grateful that he allowed me the opportunity to serve as a teaching assistant at the Woods Hole Marine Biology Laboratory, and the chance to work with gifted undergraduates in his lab.

I would also like to that the numerous lab members that I have had the privilege to overlap with over the years. I am grateful for all of the great advice and wisdom that I have received from Dr. Kathy French, Kevin Briggman, Antonia Marin-Burgin, Krista Todd, Eric Tompson, Mike Baca, Mike Baltzley, Daniel Wagenaar, and Joyce Murphy. Their thoughts and ideas are all contained within this work. I am also grateful for all of the hard work that I have received from the gifted undergraduates that I have had the pleasure to work with including, Tracy Huang, Natalie Ruiz, and Saul Copado.

I would also like to thank my thesis committee, Drs. Massimo Scanziani, Jeff Isaacson, Sascha du Lac, and Tim Gentner for their input and suggestions at all phases of this work. I feel fortunate to have had the opportunity to have so closely interacted with such acclaimed scientists. I would like to thank Massimo Scanziani and Jeff Isaacson for critical evaluations of figures and previous versions of manuscripts. I am grateful to them for stimulating discussions of my data and helping turn my often scrambled experimental ideas into their final form contained within this thesis. Their expertise has been indispensable and their enthusiasm for physiology has been inspirational.

I am also grateful to my friends and family for all of their love and emotional support during my time here at UCSD. I am particularly grateful to my wonderful wife Kate Gaudry for all of the wonderful emotional and intellectual support she has given me over the years. Scientifically, Kate has edited my manuscripts, conceived project ideas with me, and helped me profusely with programming and data representation. Emotionally, she has simply been the greatest friend and wife I could have asked for.

Chapter 2 is a preliminary draft of Gaudry Q and Kristan WB. Behavioral choice by descending inhibition of tactile sensory fibers (*in preparation*). The thesis author collected all data presented in Chapter 2. Chapter 3 includes experimental data conceived by the thesis author and collected by an additional author, Natalie Ruiz. This data is used with permission from the additional author. The work presented in Chapter 3 is being prepared for publication.

Vita and Publications

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Peer reviewed competitive journals:

Gaudry Q, Kristan WB (2008) Presynaptic inhibition of tactile sensory terminals as a mechanism of decision-making. *In preparation*.

Fortin DL, Banghart MR, Dunn TW, Borges K, Wagenaar DA, Gaudry Q, Karakossian MH, Otis TS, Kristan WB, Trauner D, Kramer RH (2008) Photochemical control of endogenous ion channels and cellular excitability. Nat Methods Apr;5(4):331-8

Cymbalyuk GS, Gaudry Q, Masino MA, Calabrese RL (2002) Bursting in leech heart interneurons: cell-autonomous and network-based mechanisms. J Neurosci 15;22(24):10580-92

Awards

2002-2004 Merck Fellowship

ABSTRACT OF THE DISSERTATION

Mechanisms of behavioral choice in the nervous system of the medicinal leech

by

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Doctor of Philosophy in Neurosciences

University of California, San Diego, 2008

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To survive, animals must execute proper behaviors at appropriate times. Animals are constantly bombarded with a plethora of sensory stimuli and must choose which of these stimuli to as important and which to ignore. By presenting an animal with two stimuli simultaneously, each of which elicits a different behavior when presented alone, we can rank-order an animal's behavioral repertoire from most preferred to least preferred. This ranking of behaviors has been termed a behavioral hierarchy. We use the leech as a model system to explore how animals place different priorities on different behaviors due to its simple nervous system, its readily identifiable neurons, and its amenability to reduced preparations. In Chapter 2 we show that feeding suppresses all competing behaviors within the leech nervous system by reducing the sensory excitation that normally drives these behaviors. Neurons involved in the ingestion phase of feeding generate a descending inhibition that travels

down the nerve cord and inhibits the tactile sensory fibers of the pressure mechanosensory neurons (P cells). We show that this inhibition is mimicked in isolated ganglia by the application of the neuromodulator serotonin. We propose that the leech uses sensory gating mediated through serotonin in the decision making process to inhibit competing behaviors during feeding. In Chapter 3 we show the distention incurred during feeding targets neurons downstream of the sensory neurons, most likely the swim gating-neurons or central pattern generating cells. Increasing levels of distention result in a gradual decrease in the number of swim cycles elicited in response to stimulation and an increase in the swim period. We propose that the feeding induced suppression of swimming is distributed within the nervous system and mediated by at least two distinct mechanisms or decision-making modules: ingestion prevents the initiation of swimming by targeting the sensory neurons that drive it, whereas distention inhibits the maintenance of swimming by targeting downstream neurons. We believe that distributed decision-making networks may be universal in the nervous systems of all complex animals.

1. Introduction

Decisions and behaviors

Animals generally express a single behavior at a time (Sherrington, 1906). Selecting the proper behavior from a plurality of options is vital to an animal's survival. Finding food, selecting a mate, and avoiding predation all require integrating different sensory cues and initiating the behavior that yields the most success.

History of the study of decision-making

The study of decision-making far predates the modern era of neurobiology (Glimcher, 2003). Ancient Greek philosophers such as Aristotle debated the origin of human decision-making. Aristotle proposed that the human soul resided in the heart and was responsible for the production of actions in response to sensation. This view separated decision making into three components (1) a physical sensory system, (2) a nonphysical entity that processed this information, (3) and a physical motor system that was responsible for carrying out the action. This view was widely held until the second century A.D. when the Greek physiologist Claudius Galen challenged the location of the human soul. Galen used observations from animal experiments to determine that the soul, or decision-making process, must reside in the nervous system. He observed that animals quickly deprived of their hearts briefly retained the ability to react to stimuli, whereas animals whose spinal cords were severed lost all ability to move. In the 17th century, René Descartes proposed that human decision-making could be viewed as two types of decisions involving two distinct processes.

The first were simple reflexive decisions, which were deterministic and predictable. These decisions had an entirely physical basis within the nervous system. The second class of decision-making strategies involved complex decisions and relied on the nonphysical soul. By attributing the first class of decisions, reflexive behaviors, to entirely physical processes, this revolutionary theory paved the way for a modern neurobiological approach to studying decision-making.

Models of decision-making

Animals make a variety of different types of decisions and thus numerous strategies have evolved to study the decision-making process. The neurobiological approach to studying decision-making can be broken down into three principle types of studies: sensory discrimination, choice variability, and choice competition. Sensory discrimination tasks force an animal to perform one behavior if a certain stimulus is presented or perceived by the animal and another behavior if a different sensory stimulus is perceived. This type of decision-making has been extensively studied in the visual saccade system of monkeys (Newsome et al., 1989; Shadlen and Newsome, 1996; Glimcher, 2001; Shadlen and Newsome, 2001; Glimcher, 2003; Schall, 2005). Monkeys can be trained to saccade to a specific target for a reward. Different components of the decision-making process can be independently manipulated to determine where those processes are taking place in the primate. The time interval between stimulus presentation and saccade initiation can be varied. For example, to determine *when* a saccade should be made versus *where* a saccade should be made (Glimcher and Sparks, 1992; Glimcher, 2001). The probability that a saccade or the

reward associated with a particular saccade can also be varied to see how prior knowledge of a task are encoded in the brain and incorporated into decision-making (Platt and Glimcher, 1999). Other model organisms may provide addition insight into how an animal chooses between two options based on sensory information. The fruit fly, *Drosophila melanogaster*, is a powerful candidate for identifying genes and molecules potentially critical for these types of decision-making tasks. *Drosophila* have recently been shown to discriminate between substrates in to order to identify optimal egg-laying sites (Yang et al., 2008).

The study of decision-making through choice variability has recently gained popularity. One method of studying choice variability is to repeatedly present an animal with an identical stimulus that can elicit multiple behaviors. By providing the same stimulus on every trial, this approach reduces the role of sensory input and allows the experimenter to focus on downstream decision-making elements. This method was combined with optical imagining in the leech to identify neurons critical for choosing whether to swim or crawl in response to a stimulus (Briggman et al., 2005). Another approach to studying choice variability in animals is to eliminate the sensory stimulus entirely and determine how animals choose behaviors at random in a sensory deprived environment (Garcia-Perez et al., 2005; Maye et al., 2007).

A final method for studying decision-making involves choice-competition. The work contained within this thesis will deal primarily with this form of decision-making. Because animals are generally thought to engage in single behaviors at a time, there must be mechanisms for preventing all other behaviors when one appropriate behavior is selected (Sherrington, 1906). By simultaneously presenting animals with

two different stimuli, each of which elicits a different behavior when presented alone, one can rank any number of behaviors into a hierarchy, from most preferred to least preferred. The ranking of responses forms a behavioral hierarchy that governs an animal's action when simultaneously presented with a variety of sensory inputs. The precise ranking of behaviors can range widely between species and can change within an animal depending on the internal or external state of the animal, such as level of satiation or the season (Davis et al., 1974b, 1974a; Gillette et al., 2000). The relatively simple nervous systems of invertebrates have been precious in determining how animals switch between behaviors, and how animals prevent the activation of conflicting behaviors once engaged in a more appropriate behavior (Kovac and Davis, 1977, 1980; Jing and Gillette, 1995; Norekian and Satterlie, 1996; Shaw and Kristan, 1997; Jing and Gillette, 1999; Gillette et al., 2000; Jing and Gillette, 2000; Norekian and Satterlie, 2001; Staras et al., 2003; Orr and Lukowiak, 2008).

Choice-competition and behavioral hierarchies were first described in the marine mollusk *Pleurobranchaea* (Davis and Mpitsos, 1971; Davis et al., 1974b). Neuronal circuits giving rise to behaviors were thought to be distinct and the initiation of behaviors were thought to be mediated through the activation of command neurons specific to each behavior (Wiersma and Ikeda, 1964; Kupfermann and Weiss, 1978; Weeks and Kristan, 1978). The popular theory at the time was that inhibition of competing behaviors was conducted at the highest levels of the circuits (Kovac and Davis, 1980; Krasne and Lee, 1988; Edwards, 1991). This hypothesis was supported by previous work on the feeding induced suppression of withdrawal responses in *Pleurobranchaea* (Kovac and Davis, 1977). During feeding, a reduction in the

amount of excitation produced in withdrawal motorneurons is observed while the animal is feeding. This inhibition arises from an interneuron involved in the feeding network, but its target has not since been identified. The putative target was considered to be a command neuron thought to be responsible for the local withdrawal reflex.

The theory that behavioral choice is regulated by competition selectively between command neurons challenged and has generally fallen out of favor (Kristan and Shaw, 1997; Kristan and Gillette, 2007). The hypothesis was directly challenged in the well-characterized circuits of the leech. In the leech, shortening and swimming are mutually exclusive (Shaw and Kristan, 1995). To swim, leeches first elongate and flatten to maximize their efficiency for moving through water. The shortening reflex prevents this behavior and, therefore is above swimming in the leech behavioral hierarchy. To test for the inhibition of competing behaviors at the level of command neurons, Shaw and Kristan recorded from various neurons in the swim circuit while delivering stimuli that generally elicit shortening behavior (Shaw and Kristan, 1997). These experiments revealed a distributed inhibition that did not specifically target the command neurons of the swim circuit. They found that three out of the four types of swim command neurons tested actually received excitation during a stimulus that elicits shortening. By characterizing the response of several neurons across a variety of behaviors, including swimming, crawling, and shortening, Shaw and Kristan concluded the networks underlying behaviors in the leech were distributed rather than dedicated, and that the decision to select between competing behaviors is also distributed and not selectively expressed at the level of command neurons (Kristan and

Shaw, 1997). This general view of how behavioral circuits interact is referred to as "reconfiguration" and has been demonstrated extensively (Rozsa, 1995; Ramirez, 1998; Lieske et al., 2000; Tazaki and Tazaki, 2000; Jing and Weiss, 2001; Nargeot, 2001; Vehovszky and Elliott, 2001; Faumont et al., 2005; Briggman and Kristan, 2006; Jing et al., 2007).

At the top of the medicinal leech behavioral hierarchy resides feeding behavior, which suppress all other behaviors. Tactile stimulation that would otherwise elicit swimming, crawling, and shortening produce only very weak local bends during feeding (Misell et al., 1998). Although this hierarchy is extremely robust behaviorally, nothing is known about the neuronal circuitry underlying this suppression. The inhibition of mechanically elicited behaviors during feeding is the central theme of this thesis.

Behavioral circuits in the leech

The experiments described in this thesis focus primarily on three behaviors -- feeding, local bending, and swimming -- and the neuronal circtuis underlying them in the medicinal leech. I will thus briefly review each behavior and its neuronal circuit.

Although little is known regarding the neuronal circuitry responsible for the generation of leech feeding behavior, much is known about the behavior itself. The medical leech, *Hirudo verbana*, is an obligate sanguivore. Hungry leeches integrate a variety of external stimuli to locate prey and determine its edibility. They combine visual and mechanonsensory signals from water waves to isolate the source of disruption in their environment (Young et al., 1981; Dickinson and Lent, 1984;

Carlton and McVean, 1993). Prior to feeding, leeches utilize a combination of swimming and crawling to locomote towards a potential prey item (Misell et al., 1998). Once the leech makes contact with a potential host, it will use thermal and chemical cues to determine if the host is a viable meal option. In the laboratory, leeches show a strong preference for feeding solutions warmed to mammalian body temperature versus room temperature (Dickinson and Lent, 1984). Leeches also contain at least two distinct sets of chemosensory receptors, one set that is located on the dorsal lip of the animal, and another set which is located internally (Kornreich and Kleinhaus, 1999). The chemoreceptors on the dorsal lip of the leech are used to determine if a food source is edible prior to ingestion. Application of bovine blood or a solution of sodium chloride and arginine to the lip elicits an appetitive response while the application of bitter substances, such as quinine or denatonium, suppresses feeding (Li et al., 2001). Once ingestion has commenced however, bitter substances applied to the lip of the animal are not capable of terminating feeding (Kornreich and Kleinhaus, 1999). An additional set of chemosensory receptors is found in the gut of the animal. These receptors monitor the quality of a meal during ingestion and can terminate a feeding bout if an appetitive feeding solution is switched to a non-edible solution in the middle of a feeding episode (Kornreich and Kleinhaus, 1999).

Leeches are episodic feeders with remarkable feeding abilities. They can gain up to twelve times their body weight in a single meal (See FIGURE 3.1B of Chapter 3) and survive up to a year between meals (Dickinson and Lent, 1984). These features of leech feeding behavior may have evolved to ensure the animals' survival during long bouts of food shortages. Leech feeding behavior critically relies on serotonin.

Serotonin levels in the nerve cords of leeches decreases drastically following a feeding episode, suggesting massive release from serotonergic cells within the CNS (Lent et al., 1991). The depletion of serotonin through injections of 5,7, D-HT or reserpine mimics satiation and bathing these animals in a serotonin solution restores their feeding behavior (Lent and Dickinson, 1984). Soaking leeches in serotonin solutions also modifies their feeding behaviors by increasing their biting response to warm surfaces and increasing the total volume of blood ingested per meal (Lent and Dickinson, 1984). Serotonergic neurons within the cephalic ganglion of the leech are excited by the application of appetitive solutions or heat to the dorsal lip of the animal (Groome et al., 1995; Zhang et al., 2000). During feeding, peristalsis of the midbody is used to distribute blood through out the gut of the animal (Wilson and Kleinhaus, 2000). As the feeding episode continues, the leech distends until the animal is satiated and ingestion is terminated. Distention is partly responsible for the termination of feeding (Lent and Dickinson, 1987; Wilson et al., 1996). The elimination of distention by cannulating the gut and removing the ingested blood drastically increases the duration of feeding episodes. Following a feeding bout, leeches show a drastic change in their behavior (Dickinson and Lent, 1984; Misell et al., 1998). Leeches become lethargic, withdraw from otherwise appetitive stimuli, and crawl rather than swim away from noxious stimuli (Dickinson and Lent, 1984; Misell et al., 1998). The transition from using predominantly swimming as a mode of locomotion to crawling following a feeding bout will be the focus of Chapter 3.

The leech local bend response is a reflexive action contained within a single segment of the leech and pulls that segment away from the site of stimulation. Local

bending is the result of a strong contraction of longitudinal muscles ipsilateral to the site of stimulation and a relaxation of longitudinal muscles contralateral to the stimulation site (Kristan, 1982). The reflex may have evolved to allow the animal to maneuver itself around objects in its environment during crawling or as an initial withdrawal from noxious stimuli before eliciting a whole body behavior such as swimming. During local bending, mechanical deformation of the skin activates the pressure mechanosensory neurons (P cells) whose terminals innervate that portion of skin (Kristan, 1982; Lewis and Kristan, 1998). Four P cells located within each segmental ganglion innervate the body of that segment. The P cells use glutamate as neurotransmitter and excite a single layer of interneurons known as the local bend interneurons (LBIs) in a feed forward manner (Baccus et al., 2000; Kristan et al., 2005). Each P cell synapses onto each of the LBIs but does so with different synaptic strengths (Lockery and Kristan, 1990b). Thus each LBI has its own receptive field. The local bend interneurons feed forward onto a layer of motor neurons that innervate the longitudinal muscles of the body wall. The motor neurons are either excitatory or inhibitory (Stuart, 1969; Ort et al., 1974). The excitatory motor neurons cause the contractions of muscles ipsilateral to the site of stimulation (Kristan et al., 1982). The inhibitory motor neurons directly inhibit the muscles contralateral to the site of stimulation and also inhibit the motor neurons that drive those contralateral muscles (Lockery and Kristan, 1990a). This lateral inhibition within the central nervous system contributes to the localized and directed response of the local bend. Recently, an additional form of inhibition has been found between the P cells and the local bend interneurons (Baca et al., 2008). This inhibition appears to be responsible for

controlling the gain of the local bend across stimulus intensities. The magnitude of the local bend is susceptible to habituation as well as to a serotonin-mediated form of sensitization (Lockery and Kristan, 1991).

The leech swim circuit, much like the local bend circuit, has been extensively characterized (Friesen, 1989; Brodfuehrer et al., 1995; Brodfuehrer and Thorogood, 2001; Kristan et al., 2005). Leeches swim by first elongating and flattening. They use a dorsal-ventral sinusoidal wave that travels from the anterior end of the animal rearward with a period of approximately 0.3-1 second in the intact animal (Kristan et al., 1974) and about 1 second in the isolated nerve cord of the animal (Kristan and Calabrese, 1976). Like the local bend reflex, swimming can be initiated by activating mechanosensitive P cells (Debski and Friesen, 1987). The P cells synapse onto the axons a pair of command-like neurons called trigger cells that initiate swimming when stimulated briefly (Tr1 and Tr2) (Brodfuehrer and Friesen, 1986b). The somata of the trigger cells are located in the cephalic ganglion of the leech and each cell sends an axon down the length of the nerve cord. Trigger cells can initiate either swimming or crawling (Brodfuehrer et al., 2008) and most likely initiate one pattern over another depending on environmental cues (Esch et al., 2002). The trigger cells directly excite a group of interneurons, the gating cells, which in turn provide excitation for the swim central pattern generator (Weeks, 1982; Brodfuehrer and Friesen, 1986a; Nusbaum et al., 1987). The central pattern generator of the swim circuit consists of mutually inhibitory interneurons that can be assigned to one of three phase groups, 0º, 120º, and 240º, where 0º is defined as the time of the dorsal contraction (Friesen, 1989). The CPG neurons produce the swim rhythm and then impose it onto the longitudinal motor

neurons of the leech. Although the leech swim CPG can operate independent of sensory feedback, stretch receptors in the body wall do influence the period and intersegmental phase lag of the swim rhythm (Kristan and Calabrese, 1976; Cang and Friesen, 2000; Bravarenko et al., 2001; Yu and Friesen, 2004). The sensory feedback from peripheral stretch receptors in the longitudinal muscles can even entrain the swim rhythm across a transected region of the nerve cord (Yu et al., 1999). Preparations consisting of isolated nerve cords with few segments of body wall attached show that stretching the body wall during ongoing swimming can increase the period of swimming. Severe stretching of the body wall can also terminate ongoing swimming bouts (Kristan and Calabrese, 1976).

Concluding remarks

The work contained within thesis addresses the question of how does an animal bombarded by sensory stimuli choose to initiate one behavior and suppress others. I have focused on how different components of the leech feeding, ingestion and distention, inhibit the neuronal circuitry underlying both the local bend reflex and swimming. I have used pair-wise intracellular recordings and paired pulse ratios to determine the sites of plasticity as the leech changes behavioral states from non-feeding to feeding. I have also utilized semi-intact preparations to determine the effects of distention on swimming rhythm in the leech. I believe that the mechanisms underlying behavioral suppression in the leech are universal and may provide insights into how other biological systems make similar decisions.

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2. Behavioral choice by presynaptic inhibition of tactile sensory terminals

Abstract

When presented with multiple stimuli simultaneously, an animal must choose which stimulus to respond to and which to ignore. In this paper, we show that, during feeding, medicinal leeches prevent the initiation of incompatible behaviors like shortening and swimming by presynaptic inhibition of mechanosensory input. Consequently, graded reflexive behaviors such as the local bend reflex are greatly reduced in amplitude. Using a semi-intact preparation, we recorded decreases in local bending as the animals started to feed. This decrease in motor output was accompanied by a decrease in the EPSP amplitudes between the pressure mechanosensory neurons (P cells) and their post synaptic targets, which include interneurons involved in local bending, shortening, and swimming. We showed an increase in the paired pulse ratios measured at these synapses during feeding, implicating a presynaptic origin for the reduction in EPSP amplitudes. Although stimulation of sensory input failed to elicit behaviors during feeding, we found that direct stimulation of the swim-gating neuron 204 restored swimming in the posterior end of the leech while the anterior end continued to feed. Finally, we show that bath application of serotonin to isolated ganglia mimics the presynaptic inhibition caused by feeding. Hence, feeding in the leech suppresses all other touch-evoked behaviors by strongly decreasing the release of transmitter at the terminals of the mechanosensory neurons.

Introduction

Seeking the neural basis of decision-making has become a productive approach in systems neuroscience. Recording from various regions of their visual systems as primates make choices about what to look at has revealed a great deal about how and where sensory perceptions lead to eye movements (Glimcher and Sparks, 1992; Shadlen and Newsome, 1996; Glimcher, 2001; Shadlen and Newsome, 2001; Krauzlis, 2005), how previous experience affects the decision-making (Platt and Glimcher, 1999), and how rewards affect the behavioral choices (Schultz, 1998). Another influence on decision-making is an animal's behavioral state. For instance, the behavioral state of a rat heavily modulates its neural responses to auditory (Hromadka and Zador, 2007), olfactory (Murakami et al., 2005), and whisker tactile inputs (Fanselow and Nicolelis, 1999). Nociceptive responses are also reduced in mammals during sleep, encounters with a predator or dominant male, feeding, and expectation (Fields, 2004). This gating of sensory information allows animals to execute appropriate behaviors at appropriate times.

A common method used for studying decision-making in invertebrates is *choice competition*. Because animals typically perform only one behavior at a time (Sherrington, 1906), an animal presented with two stimuli, each of which elicits a different behavior when presented alone, will usually perform one of the two behaviors. By presenting different pairs of stimuli, one can rank any number of behaviors into a hierarchy, from most preferred to least preferred (Davis et al., 1974; Misell et al., 1998; Bravarenko et al., 2001). Invertebrate preparations such as the sea

slug *Pleurobranchaea*, the pond snail *Lymnaea*, and the leech *Hirudo* have proven most useful in sorting out behavioral hierarchies at the cellular and network levels (Calabrese, 2003). These studies have shown that one mechanism for making choices is for neurons involved in one behavior to suppress all lower-ranking by inhibiting a variety of functionally different neurons, including command-like neurons or central pattern-generating neurons (Shaw and Kristan, 1997).

In the medicinal leech, feeding appears to be at the top of the behavioral hierarchy (Misell et al., 1998). Electrical stimuli that elicit swimming, crawling, shortening, or local bending in a quiescent leech have no effect once a leech starts to feed. We have chosen to investigate the neuronal mechanisms by which feeding suppresses all other behaviors (Fig. 1A) because the suppression is extremely robust and the leech nervous system is both readily recorded and easily manipulated. Although the neuronal circuits underlying many leech behaviors have been well characterized (Brodfuehrer et al., 1995; Kristan et al., 1995), the local bending response is particularly suitable. Local bending is a segmental reflex that pulls the skin away from the site of touch by contracting ipsilateral muscles and relaxing contralateral ones. This response is produced by a simple feed-forward circuit in which most of the participating neurons have been identified (Lockery and Kristan, 1990; Lockery and Sejnowski, 1993). Mechanosensory cells responsive to pressure (P cells) relay tactile information from the body wall to local bend interneurons (LBIs) within each segmental ganglion, and the LBIs excite motor neurons that execute the bend (Figure 1B). Because each LBI receives excitatory input from all four P cells within a segment, it responds to pressure to the skin of the entire segment. The local

bend response is directed, however, because the relative strengths of the P-to-LBI connections, as well as the LBI-to-motor neuron connections, are systematically different (Kristan et al., 1995). This simple neuronal circuit provides a useful framework for determining how the feeding network suppresses behavior. The inhibition could, in principle, be directed at the sensory, interneuronal, or motor neuronal levels. We have found that the major source of the inhibition appears to be at the synaptic terminals of the sensory neurons.

Methods

Preparations: We performed all experiments on the European medicinal leech, *Hirudo verbana* (formerly *Hirudo medicinalis*) (Siddall et al., 2007). We maintained the leeches were maintained at 15º C in artificial pond water; they weighed 0.5-1.5 grams. We anesthetized the leeches by immersing them in ice-cold leech saline solution consisting of (in mM): 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose, and adjusted to a pH of 7.4 with hepes buffer. We used only hungry leeches, defined by their exploratory behavior: raising of the head and flaring the dorsal lip in response to agitating the water surface in their aquaria. During the subsequent dissection, we confirmed that these leeches did not have a blood meal within their crop, a sure sign of hunger. We used two types of preparations: (1) single isolated ganglia from the midbody of the leech, and (2) semi-intact animals, in which the posterior nervous system was exposed. We dissected isolated ganglia free of the body wall and pinned them onto a layer of Sylgard® on the bottom of a recording chamber. For semi-intact preparations, we made a longitudinal incision from the tenth segment to the tail sucker

along the dorsal midline. We then removed the body wall, viscera, and muscles from the posterior half of the body, leaving only the nerve cord. For recording extracellular motor units, we dissected the DP nerves free from the muscle and connective tissue surrounding it. We then pinned the intact anterior portion of the animal to a layer of Sylgard® on the bottom of the chamber (Figure 1C). We pulled the nerve cord through a slit in the Sylgard® and pinned it down in the recording portion of the chamber. We inserted a plastic slide to divide the two chambers and to prevent any blood leaking into the anterior chamber from reaching the recording sites in the posterior chamber. We fed the semi-intact preparations on thawed bovine serum made by centrifuging fresh whole bovine blood (Animal Technologies, Tyler, Texas) at 2500 rpm for 60 minutes. We delivered the serum from a Falcon® tube fitted with a preserved piece of sheep intestine (sausage casing). We coaxed the leeches to feed by mechanically detaching their front suckers from the Sylgard® surface and directing it toward the feeding apparatus. Leeches began feeding as soon as their front sucker contacted the sheep intestine. During long bouts of feeding, blood was actively removed by leaving the severed portion of the gut open and superfusing away the blood that seeped into the anterior half of the chamber. In some cases, we removed the blood by cannulating the gut with PE tubing. For shorter feeding bouts, we sutured the gut shut to prevent seepage from the blood meal. To terminate a bout of feeding, we peeled the sucker from the feeding tube by inserting a coarse set of forceps between the sucker and the sheep intestine.

Solutions: We prepared all modulator solutions immediately prior to use and delivered them through a Harvard Apparatus perfusion manifold. We used serotonin hydrochloride (Crisp and Mesce, 2003, 2006), dopamine hydrochloride (Crisp and Mesce, 2004), and myomodulin (Wang et al., 1999; Masino and Calabrese, 2002) in 50 μM solutions. To facilitate diffusion of the modulators into the ganglionic neuropil, we removed the sheath surrounding the ganglion.

Electorphysiological recordings: We acquired and analyzed all data using custom Matlab software (Data Acquisition Toolbox; MathWorks, Natick, MA). We recorded extracellularly from the dorsal posterior nerves (DP) with suction electrodes fed into a differential amplifier (model 1700; A-M Systems, Sequim, WA). We stimulated the nerves (Grass® S88 stimulator) using 10 msec pulses at 20 Hz for 250 ms. We monitored the activity of just the longitudinal motor neuron cell 3 in the DP recordings by setting the trigger high enough to capture only the largest units (Ort et al., 1974). We recorded intracellularly from neuronal somata with sharp glass microelectrodes (30-45 MΩ filled with 2 M potassium acetate). We recorded from neuronal somata at 10 kHz and delivered current-clamp pulses using an Axoclamp 2B amplifier (Molecular Devices, Palo City, CA) controlled with a 16-bit analog - digital board (BNC-2090; National Instruments, Austin, TX). We identified each P and AP cell on the basis of its unmistakable soma size, soma location, and electrophysiological properties (Muller et al., 1981). We identified cell 212 by its soma position in the posterior corner of the central packet and by its contralateral bifurcating primary neurite and anterior projection out of the ganglion (Lockery and

Kristan, 1990). To determine the neuritic morphology of cell 212, we re-impaled it at the end of each experiment with a microelectrode containing Alexa 488 and injected the dye into the soma using 1 nA depolarizing pulses for 500 ms at a 50% duty-cycle. To view the dye-filled neurons, we fixed the preparation in 2% paraformaldehyde for 30 minutes and cleared it in methyl salicylate, or visualized the unfixed ganglion using a the Ziess Axioscop II microscope.

We calculated input resistances by hyperpolarizing each cell with 0.5 nA for 500ms. We determined the threshold for generating a spike in P cells (i.e., their excitability) by delivering 6 ms pulses at amplitudes from 1 to 3.6 nA in increments of 0.2 nA. To be able to detect both increases and decreases in excitability after feeding, we excluded those P cells with pre-feeding thresholds of 0.2 nA because decreases in excitability could not be detected. For the LBI 212 and the AP neurons, we calculated excitability as the number of spikes elicited during the 500 ms depolarization minus the number of spikes during the 500 ms preceding the stimulus. We normalized the values by dividing by the post-feeding excitability by the pre-feeding excitability. To determine EPSP amplitudes, we hyperpolarized the neurons to prevent their spiking during the evoked excitatory synaptic inputs. We measured the EPSP amplitudes as the maximal voltage measured within 50 ms of the sensory cell spike. If there were polysynaptic components, we used only the initial peak. To calculate the optimal paired pulse interstimulus interval (ISI) for revealing changes in probability of releases, we used a modified leech saline that contained half the normal level of calcium (0.9 mM) and twice the normal level of magnesium (3.0 mM). We measured paired pulse ratios (PPR)—the amplitude of the second evoked EPSP in the pair

divided by the amplitude of the first one--only once per minute to prevent synaptic depression.

Statistics: We used GraphPad Instat software to perform statistical tests. We used repeated measures analysis of variation (ANOVA) with Tukey *post hoc* test to compare pre-feeding, feeding, and post-feeding data. We averaged data taken during each condition within an animal and performed statistical tests on the mean values from each animal. During extracellular stimulation experiments, we stimulated each leech three times in each condition, then averaged the data to generate a single value for that animal in that condition. When comparing ganglia exposed to serotonin versus control ganglia, we used a repeated measures ANOVA with Bonferroni *post hoc* test between relevant columns. We used paired student t-tests at each time interval to compare the PPR measured in normal versus modified leech saline for that interval.

Results

Feeding decreases motor activity in response to nerve stimulation

To study how feeding inhibits somatosensory reflexes, we employed a semi-intact preparation previously used to study feeding in leeches (Wilson et al 1996). We removed the body wall and inner organs in the posterior half of a leech, leaving only the nervous system intact (Figure 2.1C, D). The anterior end of the animal was free to perform behaviors while recordings were made from the posterior segmental ganglia.

In this condition, the leeches typically remained at rest until we presented them with a tube of blood covered by a membrane of sheep intestine (Figure 2.1C, D). We stimulated a nerve that activated axons of two mechanosensory neurons, a touch (T) cell and a pressure (P) cell while the semi-intact leech were either resting or feeding. We recorded motor activity with suction electrodes on another segmental nerve.

The dorsal posterior (DP) nerve contains both the sensory afferents of the mechanosensory cells and the motor efferents of a dorsal longitudinal muscle excitatory motor neuron, cell 3, as well as several other motor neurons. We stimulated one DP nerve in midbody ganglion 15 and recorded motor activity of the contralateral DP nerve. Figure 2.2A shows typical DP nerve responses resulting from contralateral nerve stimulation during both non-feeding and feeding conditions. The stimulus consisted of a 250 ms train of five pulses, each 5 ms in duration (black bar). Although the stimulus caused an increase in spike frequency during both feeding and non-feeding conditions, the increase was less pronounced during feeding conditions (Figure 2.2B). After the nerve shock, the spike rate decreased during both the feeding and non-feeding conditions, but the post-stimulus spike rate during feeding conditions remained lower than during non-feeding.

For each leech, we presented the stimulus and recorded spikes three times before initiating feeding (i.e., the first non-feeding session). We then got the leech to feed and presented three stimuli during the first feeding session. We continued with three additional sets of recordings during a second non-feeding session (after the leech's sucker was peeled from the membrane) for eleven leeches. In five of these eleven leaches, we recorded a second feeding session and a third non-feeding session.

Following each stimulus, we counted the number of spikes elicited during the five seconds following the stimulus and averaged this number across stimuli within each session. On average, the post-stimulus spike count during feeding sessions was 45% lower than during non-feeding sessions (Figure 2.2C; $p < 0.001$, repeated measures ANOVA, $n = 11$ for the first feeding session and $n = 5$ for the second feeding session).

Feeding decreases sensory input into the leech ganglion

To determine how feeding suppresses local bending, we looked for changes in both synaptic and intrinsic properties in the P cells and the LBIs. We collected data during three-minute periods before, during, and after feeding. We measured responses to P cell bursts and excitability measurements just once during the pre-feeding and feeding phases to avoid synaptic depression and behavioral habituation. Post-feeding values were taken as the last 9 recordings made after the EPSPs stabilized (6.6 ± 1.0 minutes for a local bend interneuron, LBI 212, and 7.5 ± 1.1 minutes for the AP neuron after the termination of feeding).

To determine how feeding affects mechanosensory cell synaptic transmission, we stimulated P cells to fire a single action potential every twenty seconds while recording EPSP amplitudes from LBI 212, along with simultaneous recordings from the DP nerves to monitor motor output (Figure 2.3A). We also recorded the synaptic potentials made by the P cell onto the AP neuron to determine if changes were restricted to the local bend circuit, since the AP neuron receives monosynaptic input from the P cell but is known not to be involved in the local bend circuit. Every three

minutes, we produced a train of action potentials (a burst) in the P cell to mimic a more natural stimulus (Figure 2.3B). This burst of sensory spikes reliably elicited a local bend response within the DP nerves.

To test for changes in intrinsic excitability of the P cells, the spike threshold was determined by delivering brief 5 ms pulses of increasing current into the P cells until an action potential occurred (Figure 2.3C, black trace). This was measured every three minutes immediately following the P cell burst stimulus. Because LBIs and the AP neurons are tonically active at rest, their intrinsic excitability was determined by injecting 0.5 nA for 500 milliseconds into the soma of the neuron. To determine the excitability of these neurons, we subtracted the number of action potentials in the 500 ms prior to the stimulus from the number elicited during the stimulus (Figure 2.3D).

Neither the resting membrane potential nor the input resistance changed between feeding and non-feeding conditions for any of the neurons tested. Only the AP neuron showed a significant decrease in excitability during feeding (18%, data not shown). These results suggest that the intrinsic properties of these neurons are not responsible for the decrease in local bending during feeding.

Figure 2.4A shows that a 50% reduction in EPSP amplitude occurred within the first twenty seconds of feeding and was sustained throughout the feeding episode. The feeding-induced synaptic depression was seen in both the LBI 212 (black trace, Figure 2.4B left, $p < 0.001$ repeated measures ANOVA, n=10) as well as the AP neuron (grey trace, Figure 2.4B right, $p < 0.001$ repeated measures ANOVA, n=10), suggesting that all targets of the P cell are affected by feeding and not just those involved in generating local bends. Feeding also depressed the polysynaptic

connection between the P cells and the Retzius neurons in response to bursts elicited in the P cell (data not shown). Monitoring the DP nerve activity during both single and bursting P cell stimulation showed similar results to those previously described (Fig. 2.2): during feeding, single P cell spikes elicited fewer DP nerve spikes (for ipsilateral nerve, $p < 0.05$) as did bursts of P cell spikes (for ipsilateral nerve $p < 0.001$ and for contralateral nerve $p < 0.01$).

Feeding decreases the EPSP amplitudes observed between P cells and interneurons involved in shortening and swimming

In order to further show that the reduction in sensory input was not restricted to the local bend circuit we repeated our previous experiment with interneurons involved in the shortening and swimming circuit of the leech. The S cell is a readily identifiable interneuron found in every leech segmental ganglion and receive direct excitation from the P cells within it's own ganglion (Gardner-Medwin et al., 1973). The S cell's activity is correlated with shortening behavior and it is necessary for inducing various forms of non-associative learning in the shortening reflex (Sahley, 1995). The ablation of single S cell axons can prevent sensitization of the shortening reflex and the ability to sensitize returns only once this axon regenerates (Modney et al., 1997). During feeding, we observed a 48% decrease in EPSP amplitude ($p < 0.05$, $n = 5$, mean initial EPSP amplitude = 2.43 mV). This synaptic depression is similar in magnitude to the depression observed at the P to LBI synapse and may explain in part why less shortening is observed during leech feeding.

The first interneurons to receive monosynaptic excitation in the swim circuit are the trigger cells; Tr1 and Tr2 (Brodfuehrer and Friesen, 1986a). The trigger cells are located in the sub esophageal ganglion (head brain) and project an axon down the length of the leech nerve cord and excite swim interneurons within the segmental ganglia (Brodfuehrer and Friesen, 1986b). Trigger cells also have command-like properties. Brief depolarization of the trigger cells is sufficient to elicit prolonged bouts of swimming in the isolated leech nerve cord (Brodfuehrer and Friesen, 1986c). Because the trigger cells are located in the head brain of the leech, we could not record from using out standard semi-intact feeding preparation. Instead, we used an isolated lip preparation in which appetitive stimuli could be administered to the lip to induce fictive feeding while stimulating and recording from mechanosensory and trigger neurons respectively (Figure 2.5A). We defined fictive feeding as the activation of a pharyngeal motor neuron (LL cell) in response to blood serum application to the lip (data not shown)(Lent and Dickinson, 1984). P cells in the head brain neuromere 1 were while EPSP's were recorded in the contralateral Tr1. Tr1 showed no endogenous changes in its membrane properties while the P cells of the head brain showed a 3.9 mV hyperpolarization in response to blood serum application to the lip ($p < 0.05$, $n = 5$, mean Rm before serum application = -32 mV). When P cells were driven to fire a train of action potentials, we observed a large depolarization in the post-synaptic Tr1 (Figure 2.5B). This depolarization was reduced by 36% when serum was applied to the lip (Figure 2.5C, $n = 5$, $p < 0.01$). Single EPSP amplitudes decreased by 44% with serum application (Figure 2.5D, $n = 5$, $p < 0.01$).

Feeding causes a decrease in P cell probability of release

To test whether there was a presynaptic contribution to the synaptic depression observed during feeding, we measured paired-pulse ratios (PPR) in the P-AP synaptic potential. Any change in PPR is taken as a strong indication that presynaptic release has been modified (Schulz et al., 1995; Kleschevnikov et al., 1997; Santschi and Stanton, 2003). To use this measure, we first needed to determine the interstimulus interval (ISI) between P cell spikes for detecting the largest change in presynaptic probability of release (P_r) at this synapse. Figure 2.6A shows that an ISI of 500 ms produced the largest difference between PPRs recorded in normal saline (grey diamonds) and in a modified saline solution (Mg^{++} increased to 3 mM, Ca^{++} decreased to 0.9 mM) (black squares) designed to reduce the probability of release. The PPR is larger when the release is made smaller in the modified saline, in accordance with expectations (Nicholls and Purves, 1972). An ISI of 500 milliseconds was thus used throughout later experiments to reveal changes in P_r at P cell synapses.

In semi-intact leeches, feeding caused a rise in PPR during synaptic depression (Figure 2.6B). We calculated PPRs by measuring the EPSP amplitudes in response to a first P cell spike (Figure 2.6B; black) and a second P cell spike (Figure 2.6B; grey) every minute at the P cell to AP neuron synapse. Both synaptic potentials decrease during feeding, but the first EPSP amplitude decreases more than the second, resulting in an increase in the PPR (Figure 2.6B; dashed black curve). Inserts show sample traces of each EPSP in pairs of pulses taken before, during, and after feeding. Figure 2.6C shows the average EPSP amplitude in response to the first P cell spikes and the

average PPR in the different conditions. The EPSP amplitude significantly decreases ($p < 0.001$, $n = 9$) during feeding, and the PPR significantly increases ($p < 0.01$, $n = 9$) during feeding. Paired pulse ratios were also taken at the P to trigger cell synapse with the same stimulation protocol but using out isolated lip preparation. We observed a 73% increase in the PPR at this synapse ($p < 0.05$, $n = 5$)(data not shown).

Stimulating gating neurons elicits swimming motor program during feeding

Because feeding suppresses all behaviors in the leech (Misell et al., 1998) and only the presynaptic terminals of the mecahnosensory neurons were affected by feeding (Figures 2.4, 2.5, 2.6), we wanted to determine whether the interneurons responsible for activating and patterning other behaviors would also be unaffected by feeding. We used the well-characterized leech swim circuit (Brodfuehrer et al., 1995; Kristan et al., 2005) because swimming is readily initiated by the activation of single neurons (Weeks and Kristan, 1978; Brodfuehrer and Friesen, 1986c). The trigger cells Tr1 and Tr2 are located in subesophageal ganglion of the leech, so they are not accessible during feeding in our semi-intact preparations. We thus aimed to stimulate the gating neuron, cell 204, which connects the trigger cells to the swim central pattern generator (Nusbaum et al., 1987). Conveniently, cell 204 is readily found in ganglia 10 through 16 and provides a tonic excitation shown to initiate and maintain swimming bursts in both isolated nerve cords and semi-intact preparations (Weeks & Kristan, 1978). We tested whether cell 204 could elicit swimming in semi-intact leeches as they fed (Figure 2.7A). We impaled a single cell 204 in segments 11-14 while the

anterior end of the animal fed from bovine serum. We monitored swimming activity using nerve recordings from ganglia both anterior and posterior to the impaled cell 204 to be sure that swimming propagated along the nerve cord in both directions. DP nerve recordings monitor the dorsal contraction phase of swimming and AA:B1/B2 nerve recordings monitor the ventral contraction phase. Stimulation of a single cell 204 was sufficient to initiate the swimming motor program (Figure 2.7B). Each swim episode lasted as long as cell 204 remained depolarized. In all 4 that produced swimming in response to injecting 1-2 nA of depolarizing current into cell 204 depolarization before feeding, the same depolarization produced swimming during feeding (Figure 2.7C). The swimming activity was normal both in the anterior to poster delays (compare bursts in DP(G10) to those in DP (G15)) and in the alternation between dorsal and ventral contractions (compare bursts in DP(G15) to those in AA:B1/B2 (G15)). These data show that CPG interneurons and their connections to the motor neurons remain capable of generating normal swimming rhythm even while the anterior end of the animal is engaged in feeding behavior.

Serotonin may be responsible for the feeding induced suppression of local bending

Several neuromodulators were tested for their abilities to mimic the effects of feeding. Among the modulators tested were dopamine (50 μM), myomodulin (50 μM), and serotonin (50 μM). The modulators were tested for their ability to suppress motor activity and depress the P to AP synapse with an increase in PPR as was

observed during feeding. Among the modulators tested serotonin was the only one to reliably meet these criteria.

Extracellular nerve recordings were made from the DP nerve to measure the effect of serotonin on the local bend motor output (Figure 2.8A). A train of five P cell spikes was initiated while motor units were recorded. Motor units were summed within five seconds of the stimulus in a manner similar to that of Figure 2. Serotonin decreased the amount of motor units generated in the nerves ipsilateral to the P cell by 81% and by 78% compared to control ganglia (Figure 2.8B; $p < 0.001$. Serotonin application $N = 12$, control saline application $N = 4$).

Serotonin also presynaptically depresses the EPSP amplitudes at the P to AP synapse (Figure 2.9A). Serotonin decreased the mean first EPSP amplitude to 42% of its original size ($p < 0.001$, $n = 8$). The effect of serotonin on EPSP amplitudes was generally long lasting and could not always be reversed by washout. Control ganglia were employed to rule out the possibility that general run down of the preparation was responsible for the synaptic depression. These ganglia were matched for similar initial EPSP amplitudes ($10.6 \pm .88$ mV control vs. $10.4 \pm .82$ experimental). These ganglia received the same P cell stimulation protocol but were never exposed to serotonin. Ganglia exposed to serotonin had significantly lower EPSP amplitudes than control ganglia (Figure 2.9B; $p < 0.001$, $n = 8$). Paired pulse ratios were also continuously monitored at 1 minute intervals and increased by 65% percent during serotonin application (Figure 2.9C; $p < 0.001$, $n = 8$). Paired pulse ratios were also larger than those measured in control ganglia ($p < 0.001$).

Discussion

As previously described (Dickinson & Lent, 1984; Misell et al., 1998), we have found that medicinal leeches do not respond even to strong mechanical stimulation while they are feeding. Because many touch-evoked behaviors (e.g., local bending, swimming, crawling, and whole-body shortening) are elicited by activation of P cells (Kristan et al., 1982), we focused on the synaptic contacts made by P cells onto interneurons involved in these behaviors. We found that a major change produced by feeding was a significant (50% or more) reduction in the synaptic potentials made by each P cell onto its target neurons: cell 212, a local bend interneuron (Lockery & Kristan, 1990); the S cell, involved in whole-body shortening (Sahley et al., 1994); the Tr1 cell, which triggers swimming (Brodfuehrer & Friesen, 1986b); and the AP cell, of unknown function (Wessel et al., 1999). The synaptic connections from P cells onto the AP cell and the Tr1 cell showed an increase in their paired-pulse facilitation ratios during the feeding-induced depression (Fig. 2.6), suggesting that, in both cases, the mechanism of synaptic depression is a decrease in the probability of release at the P cell terminals (McNaughton, 1982; Kleschevnikov et al., 1997).

Role of presynaptic inhibition in behavioral choice

To determine whether presynaptic inhibition could account for *all* the behavioral effects seen during feeding, one would need to show that: (1) the documented synaptic changes are sufficient to turn off all the behaviors, and (2)

feeding does not affect other neuronal properties. Both of these conditions are extremely difficult to satisfy definitively, but we have evidence that both are true.

(1) The documented synaptic changes are sufficient to turn off all behaviors. Misell et al. (1998) reported that the only behaviors elicited by even strong tactile stimulation during feeding were "small local bends at the stimulus site or no response at all". Our results (Fig. 2) show that motor neuronal responses characteristic of local bending were reduced by 30-50%, and that there were no motor activity patterns that would indicate any other behavior. Because the relationship between motor neuronal firing and muscle contraction is nonlinear, this decrease in firing rate would produce an even greater decrease in muscle contraction (Mason and Kristan, 1982).

We have also found that feeding suppresses swimming, a behavior with a higher threshold than local bending (Kristan et al., 1982). The swim-initiating pathway is more complex than the local bending circuit. In the swim circuit, mechanosensory neurons excite trigger neurons, which excite swim-gating neurons, which activate pattern-generating neurons that impose their rhythmic activity on the motor neurons (Brodfuehrer & Friesen, 1986a; Kristan et al., 2005). We tested one of the first steps in this pathway—the excitation of a trigger neurons (cell Tr1) by a mechanosensory neuron (P cell)—and found that feeding elicited a similar presynaptic inhibition of the sensory terminals. This produces a smaller response at the next step, the gating neurons.

(2) Feeding does not affect other neuronal properties. We found no changes in the properties of the interneurons produced by feeding, nor any obvious increase in the inhibitory inputs onto them. The fact that a gating neuron, cell 204, can elicit swimming in the back of the animal during feeding suggests both that the act of feeding does not affect the interactions downstream of the gating neurons (i.e., the pattern generators or the motor neurons) and, in return, the swimming pattern generator does not interfere with feeding.

Presynaptic inhibition as a mechanism for suppressing behaviors

One of the most common functions of presynaptic inhibition is the modulation of tactile sensory input in many animals, both invertebrate (Clarac and Cattaert, 1996) and vertebrate (Rudomin and Schmidt, 1999; Millan, 2002), particularly input from proprioceptors and nociceptors. The functions of presynaptic inhibition onto proprioceptors vary from controlling the gain of sensory feedback (Burrows and Matheson, 1994; El Manira et al., 1997; Rudomin, 2002; Rudomin et al., 2007; Rudomin and Lomeli, 2007), to spatial discrimination (Levine and Murphey, 1980), maintaining directional sensitivity (Blagburn and Sattelle, 1987), and central integration of sensory messages (Pearson and Goodman, 1981). In all these cases, presynaptic inhibition acts as a sensory gate, eliminating the influence of sensory input onto motor neurons or motor circuits. In addition, presynaptic inhibition can be used to modify the relative strength of inputs from projection neurons that select different

behaviors, thereby contributing directly to a behavioral choice (Beenhakker et al., 2007).

Nociceptive input is particularly susceptible to gating mechanisms. A variety of external stimuli and behavioral states, acting via brain stem pathways, can inhibit first order nociceptive inputs both presynaptically and postsynaptically to profoundly affect a mammal's response to painful stimuli (Fields, 2004; Fields, 2007). A stimulus as mild as ingesting a sucrose solution can activate anti-nociceptive pathways in rats (Segato et al., 1997) including the dorsal raphe nucleus (Miyase et al., 2005). Similarly, human infants show a reduced pain reaction in response to heel lancing while feeding on a sucrose solution (Bucher et al., 1995). More intense stimuli, such as the presence of a predator, can also produce a serotonin-mediated analgesia in mice (Kavaliers and Colwell, 1991). The "do not respond to pain" decision may activate OFF cells of the rostral ventromedial medulla (RVM) that lead to pain suppression at the spinal cord level (Fields, 2007). These decisions to ignore pain allow animals to perform other behaviors that may be more important at that time, such as feeding, avoiding predators, or seeking a mate.

In addition to being an efficient mechanism for suppressing responses to tactile stimuli, presynaptic inhibition also makes interneurons available for other behaviors. In the spinal cord of a cat, for instance, the same interneuron can be active in locomotion, in reflexive movements, and in voluntary leg movements (Jankowska, 2008). Gating out sensory input would keep the interneurons available for higher-

order commands. In the leech, the motor neurons used in local bending are also used to drive peristaltic movements along the animal during feeding (Wilson and Kleinhaus, 2000), a behavior that would be compromised if the motor neurons were affected by tactile input. In fact, there is increasing evidence that many leech interneurons contribute to more than one behavior, such as swimming and shortening (Shaw and Kristan, 1997; Esch et al., 2002; Briggman and Kristan, 2006). Hence, it would be difficult to bias one behavior without affect others. Interestingly, there is an interneuron type recently postulated (Baca et al., 2008) that might prove to be a target for sensory input. This GABAergic interneuron is driven by tactile sensory input and inhibits all local bend interneurons, probably as a way to set the gain of local bending. If feeding activated such an interneuron, it would help to turn down the local bending response.

Potential mechanisms for synaptic inhibition

We propose that serotonin is made available during feeding and that the presynaptic terminals of the P cells contain serotonin receptors, probably ionotropic, that respond to the serotonin released by feeding. Serotonin has previously been implicated as being both sufficient and necessary for the expression of feeding behavior in the leech (Lent and Dickinson, 1984). Globally depleting serotonin using the 5,7-DHT causes hungry leeches to lose interest in food, and applying serotonin to such an animal restores its appetitive behaviors (Lent, 1985). During bouts of feeding, serotonin is secreted from the serotonin containing neurons of the cephalic ganglion

and midbody ganglia (Lent et al., 1991). The serotonergic pair of giant Retzius neurons initially increase their firing rates when the leech's dorsal lip touches a tasty or warm solution (Lent and Dickinson, 1984; Zhang et al., 2000). Leech P cells have both ionotropic (Ali et al., 1998) and metabotropic (Catarsi and Drapeau, 1997) serotonin receptors. The ionotropic receptor is inhibitory and flux chloride. This receptor could provide an inhibitory shunt at presynaptic terminals. Ionotropic receptors act quickly, which is consistent with the rapid onset of synaptic depression that we observed during feeding. We have also recorded decreases in synaptic potentials (P cells to AP neurons) and motor output in isolated leech ganglia when serotonin is bath applied (Figure 2.8), accompanied by an increase in PPR during serotonin application (Figure 2.9). These results suggest that serotonin mimics feeding by suppressing motor activity by decreasing the probability of release at the P cell terminals.

Acknowledgements

This chapter is being prepared for publication as Gaudry Q and Kristan WB. Behavioral choice by descending inhibition of tactile sensory fibers (*in preparation*). The thesis author is responsible for all of the data contained within this chapter.

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Fig. 2.1: Leech behavior and the semi-intact preparation. **A**. Feeding suppresses all motor behaviors in the medicinal leech. Black circles represent sites of suppression. **B**. Schematic diagram shows connections within the local bend circuit, consisting of pressure mechanosensory neurons (P), local bend interneurons (LBI), inhibitory motor neurons (iMN), and excitatory motor neurons (eMN) (v = ventral, d = dorsal). **C.** Drawing of a semi-intact leech feeding with stimulating and recording suction electrodes on DP nerves of ganglion 15. Two intracellular electrodes are also shown directed at ganglion 15. A plastic divider separates the anterior behavioral chamber from the posterior physiology chamber. The front end is fully intact and pinned down in denervated pieces of skin at the cut posterior end. The exposed nerve cord traverses through a slit between chambers. Petroleum jelly fills any gaps in the barrier between the chambers. Blood is presented in a 15 ml Falcon tube, with its open end covered by a piece of sausage casing made from sheep intestine. **D**. Pictures of a semi-intact preparation attached to the bottom of its recording chamber by its front sucker (upper picture) and attached to the sheep intestinal membrane as it feeds (lower picture).

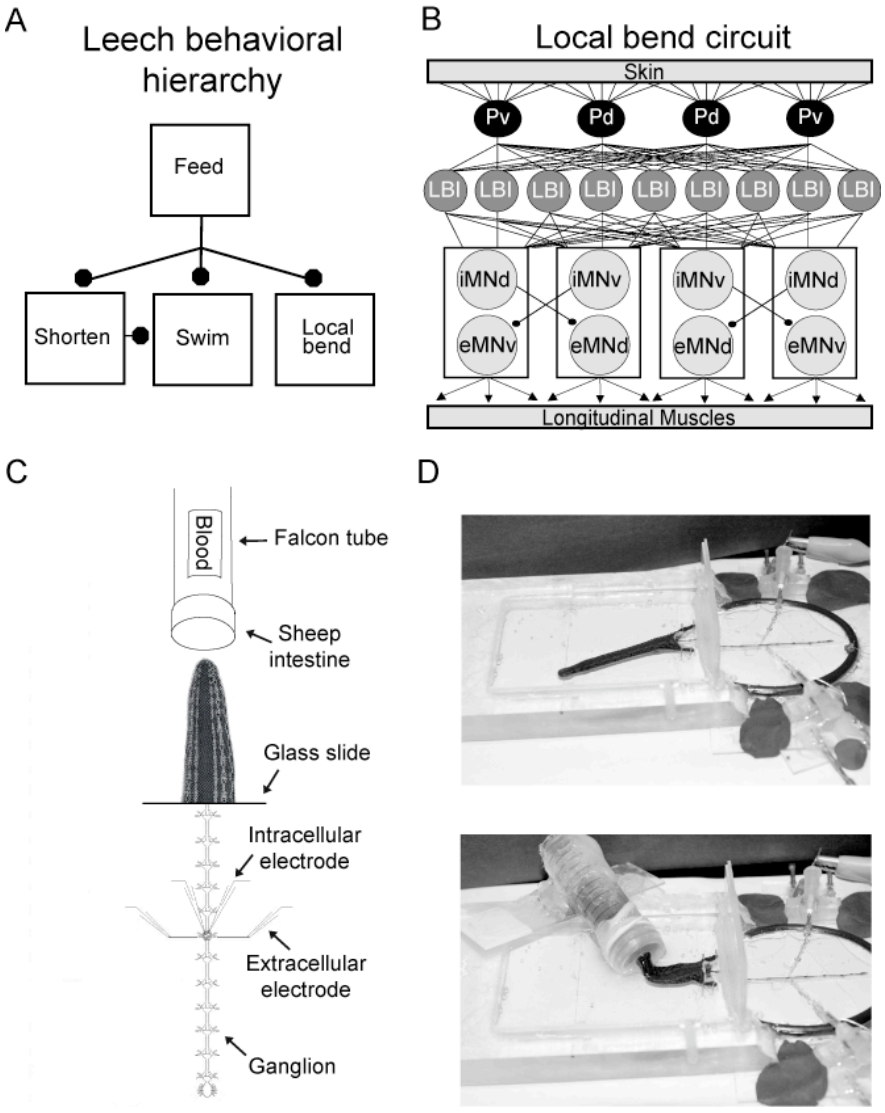

A
Leech behavioral hierarchy
Feed
Shorten
Swim
Local bend
B
Local bend circuit
Skin
Pv
Pd
Pd
Pv
LBl
iMNd
iMNv
iMNv
iMNd
eMNv
eMNd
eMNd
eMNv
Longitudinal Muscles
C
Blood
Falcon tube
Sheep intestine
Glass slide
Intracellular electrode
Extracellular electrode
Ganglion
D

Fig. 2.2: Local bend motor responses during feeding and non-feeding. **A**. Sample traces of DP nerve in response to contralateral nerve stimulation during non-feeding and feeding. Motor units were counted as any spike that crossed threshold (horizontal lines). The stimulus lasted 250 ms and was delivered every three minutes. **B**. Firing frequency of the DP motor units during feeding and non-feeding. Nerve stimulation occurred at 0 seconds. Binning was performed at 250 ms intervals. **C**. Histogram of normalized spike counts in three sessions of non-feeding and two sessions of feeding. The responses in each leech were normalized to the mean of three responses in the first non-feeding trial for that animal. One of the DP nerves in each animal was shocked three times in each condition and the mean of these trials make up each bar (*** = $p < 0.001$, repeated measures ANOVA. $N = 11$ for the first feeding session and $N = 5$ for the second feeding session. The same symbols are used throughout subsequent figures).

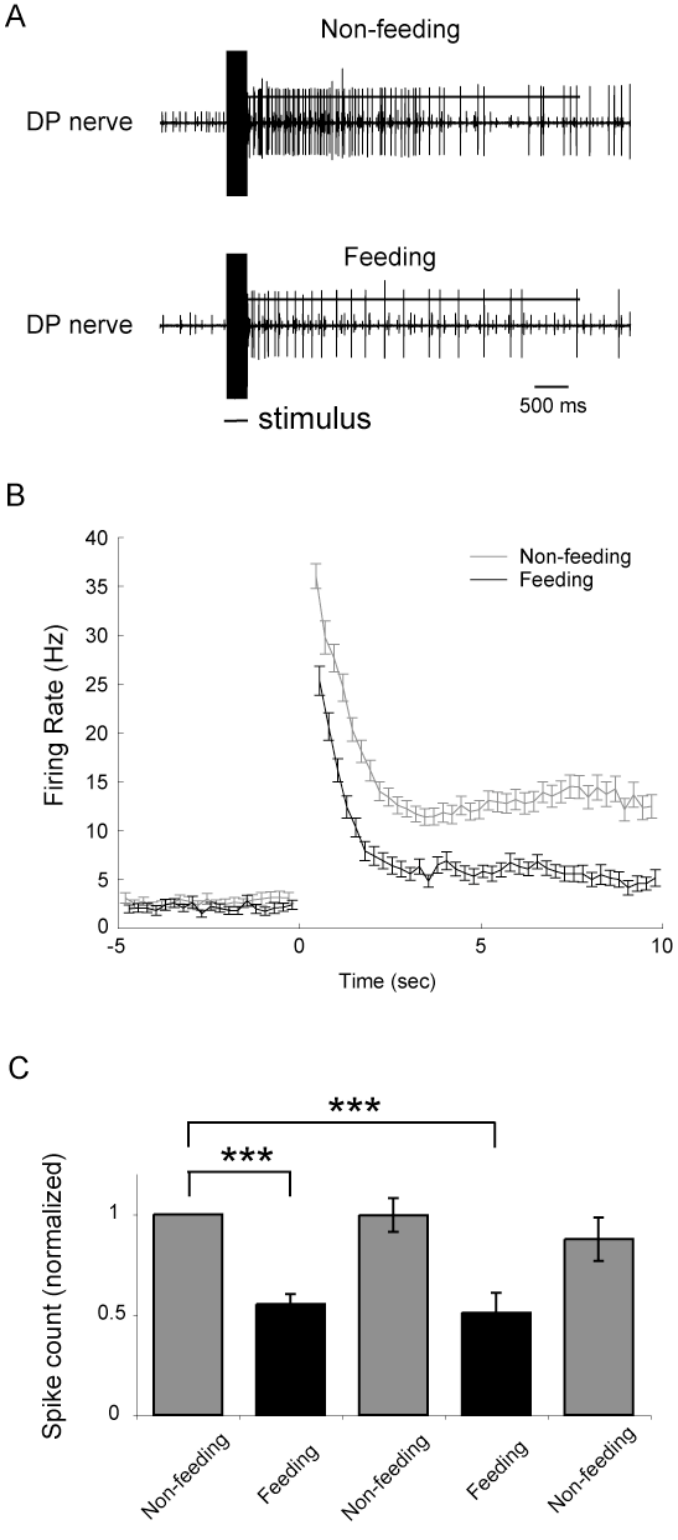

A
Non-feeding
DP nerve
Feeding
DP nerve
500 ms
stimulus
B
Non-feeding
Feeding
Firing Rate (Hz)
Time (sec)
C
***
***
Spike count (normalized)
Non-feeding
Feeding
Non-feeding
Feeding
Non-feeding

Fig. 2.3: Stimuli used to determine the site of plasticity within the local bend circuit. **A**. Sample traces of the responses of the AP neuron and the DP nerves to a single P cell action potential. The AP neuron was hyperpolarized to prevent the generation of action potentials and to determine its input resistance. The P cell was also hyperpolarized to determine its input resistance. The dorsal motor neuron activity was characterized as the largest motor unit in each DP nerve. This stimulus was delivered every 20 seconds to monitor cell activity and EPSP amplitudes. **B**. Same as in panel A, except that each P cell was stimulated to fire a burst of 5 action potentials. This results in a large local bend motor neuron burst in the DP nerves and a large compound EPSP in the AP cell. This stimulus was delivered every 3 minutes. **C**. To determine the P cell spike threshold, current injections of increasing amplitudes were injected in the somata until spiking occurred (black trace). Current level started at 1 nA and rose to a maximum of 3.6 nA at 0.2 nA intervals. **D.** To determine the excitability of each postsynaptic cell, a steady 0.5 nA current injection was sustained for 500ms. The difference in the number of spikes during these 500 ms and the 500 ms preceding the current onset was taken to be the number of spikes elicited by the current injection.

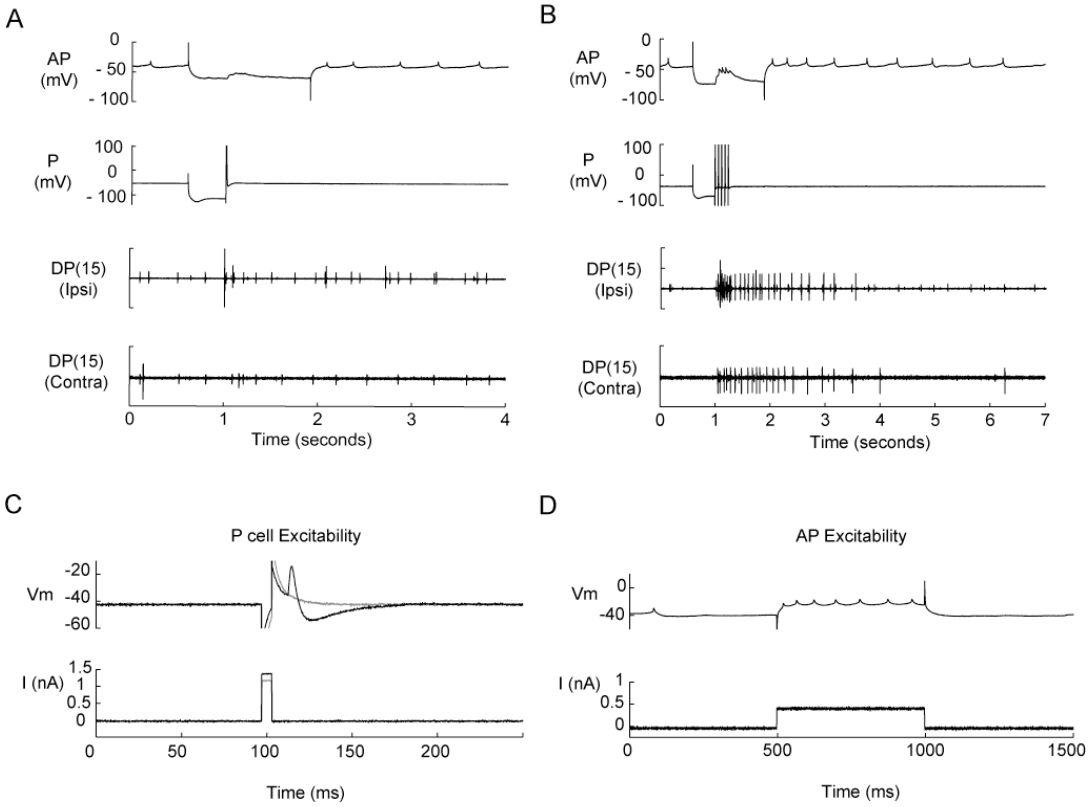

A
AP (mV)
0
- 50
- 100
P (mV)
100
0
- 100
DP(15) (Ipsi)
DP(15) (Contra)
0 1 2 3 4
Time (seconds)
B
AP (mV)
0
- 50
-100
P (mV)
100
0
- 100
DP(15) (Ipsi)
DP(15) (Contra)
0 1 2 3 4 5 6 7
Time (seconds)
C
P cell Excitability
Vm
-20
-40
-60
I (nA)
1.5
1
0.5
0
0 50 100 150 200
Time (ms)
D
AP Excitability
Vm
0
-40
I (nA)
1
0.5
0
0 500 1000 1500
Time (ms)

Fig. 2.4: Feeding decreases EPSP amplitudes from the P cell to LBI 212 and AP neurons. **A**. Time plot of EPSPs between the P cell and the LBI 212 neuron (black trace) and the AP neuron (grey trace). P cell spikes were elicited every 20 seconds. Recordings were made for 3 minutes in the pre-feeding and feeding states (grey shaded box). Post-feeding values are the last 9 recordings made after EPSPs stabilized. *Insert*, sample traces of the EPSP in the LBI 212 elicited by P cell action potentials during the pre-feeding, feeding, and post-feeding states. Scale bars = 2 mV and 100 ms. The initial peaks were used to calculate EPSP amplitudes; polysynaptic components were ignored. **B**. Summary data for changes in EPSP amplitudes measured in LBI 212 and the AP neuron (*** = $p < 0.001$, N = 10 for each type of neuron).

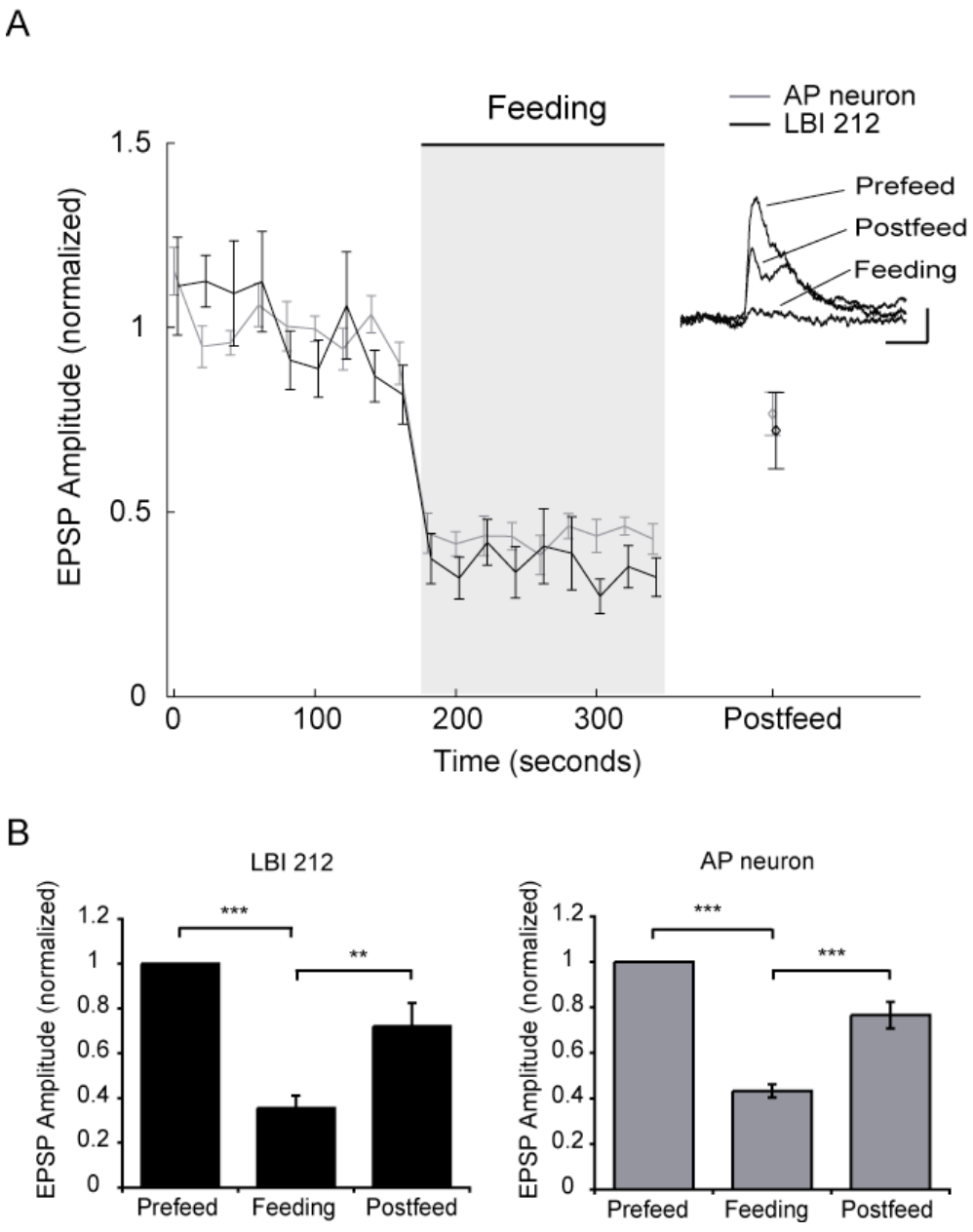

A
Feeding
AP neuron
LBI 212
Prefeed
Postfeed
Feeding
EPSP Amplitude (normalized)
1.5
1
0.5
0
0
100
200
300
Postfeed
Time (seconds)
B
LBI 212
AP neuron
EPSP Amplitude (normalized)
***
**
***
***
1.2
1
0.8
0.6
0.4
0.2
0
Prefeed
Feeding
Postfeed

Fig. 2.5: Application of blood serum to the lip of semi-intact preparations decreases EPSP amplitudes at the P cell to trigger cell synapse. **A.** A schematic diagram showing the semi-intact preparation used to test the P cell to trigger cell synapse. The diagram shows an isolated lip connected to the CNS via peripheral cephalic nerves. Blood serum and saline solutions were alternatively perfused onto the lip. **B.** Recordings of EPSP amplitudes were measured between contralateral pairs of trigger cell and P cells in the first neuromere of the sub esophageal ganglion. Bursts of P cell action potentials produce less total depolarization in Tr1 when blood serum is applied to the lip of the preparation. Black dots below traces show when P cell action potentials occured. **C.** The total depolarization of trigger cells in response to P cell bursts showed a 36% decrease when blood serum was applied to the lip of the semi-intact preparation ($n = 5$) and returned to 88% of its original value when returned to normal leech saline ($n = 5$). **D.** The EPSP amplitudes measured in response to single P cell spikes showed a 44% decrease in response to blood serum application ($p < 0.01$, $n = 5$) and returned to 84% of their initial values upon washout of serum solution at the lip ($n = 5$).

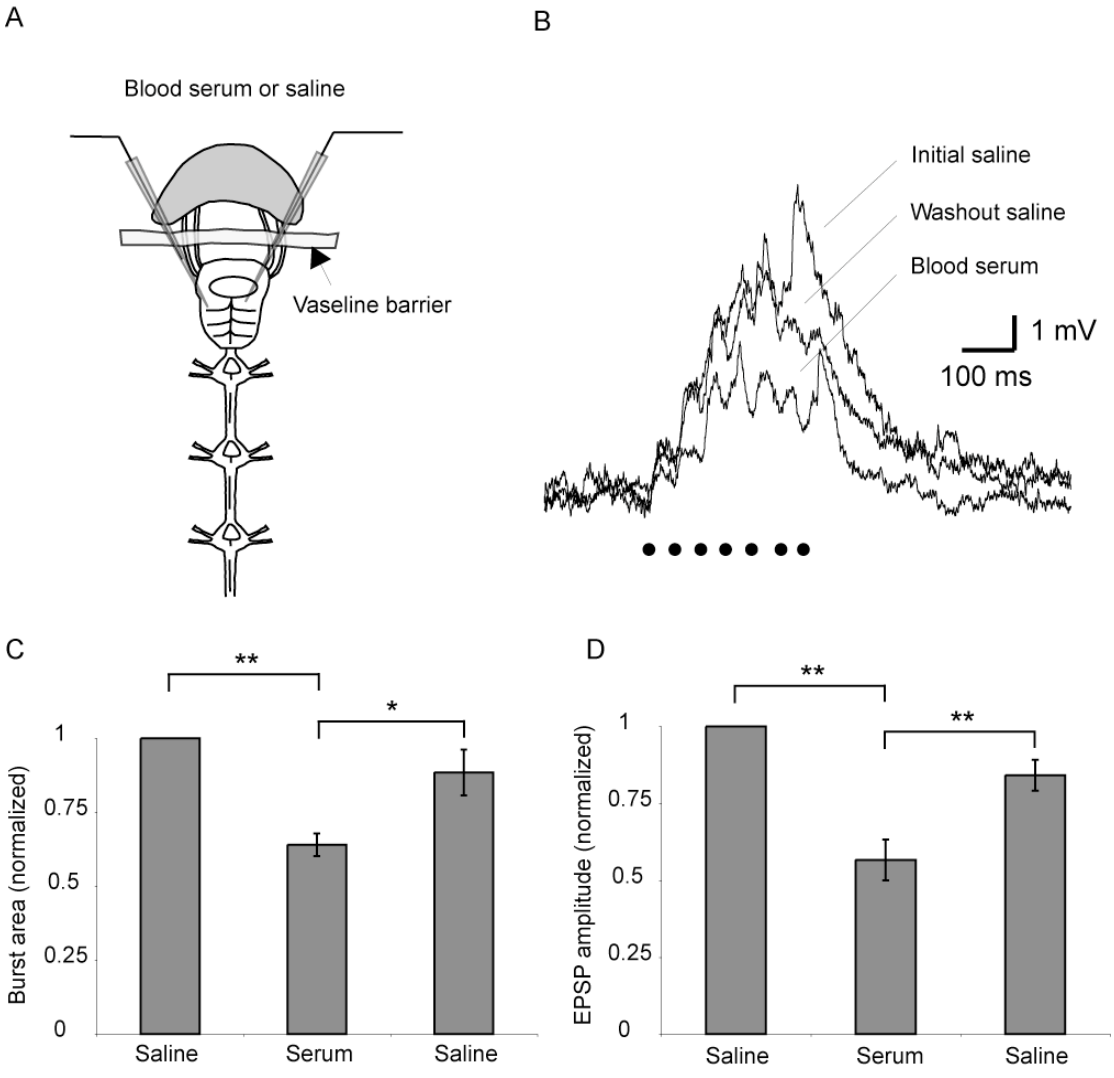

A
Blood serum or saline
Vaseline barrier
B
Initial saline
Washout saline
Blood serum
1 mV
100 ms
C
Burst area (normalized)
1
0.75
0.5
0.25
0
**
*
Saline
Serum
Saline
D
EPSP amplitude (normalized)
1
0.75
0.5
0.25
0
**
**
Saline
Serum
Saline

Fig. 2.6: An increase in PPR suggests a decrease in the probability of release at P cell terminals during feeding. **A**. A modified saline, containing 0.5X calcium and 2X magnesium and known to decrease the probability of release in leech neurons (Henderson et al., 1983) produces an increase in PPR at several of the interstimulus intervals tested. Paired student t-tests were conducted at each interval between PPRs measured in normal (grey diamonds) and modified saline (black squares). A 500 ms interval results in the largest difference in ratios ($p = 0.0064$, $n = 6$) and was used in all later paired pulse experiments. **B**. A time plot showing the effects of feeding on EPSP amplitudes and PPR. First and second EPSP amplitudes of each paired pulse are shown in black and grey respectively. The ratio between the responses to the two pulses (second/first) is shown as a dashed black curve. Inserts show sample traces from one such experiment, with the same conventions: black = first response, grey = second response. Scale bar = 2mV and 50 ms. Measurements were taken every minute for 4 minutes in each condition. Post-feeding values were calculated as the mean of the last 4 recordings made after EPSPs stabilized (5.3 ± 0.66 minutes after the termination of feeding). **C**. Summary data for changes in first EPSP amplitude and PPR measured during feeding.

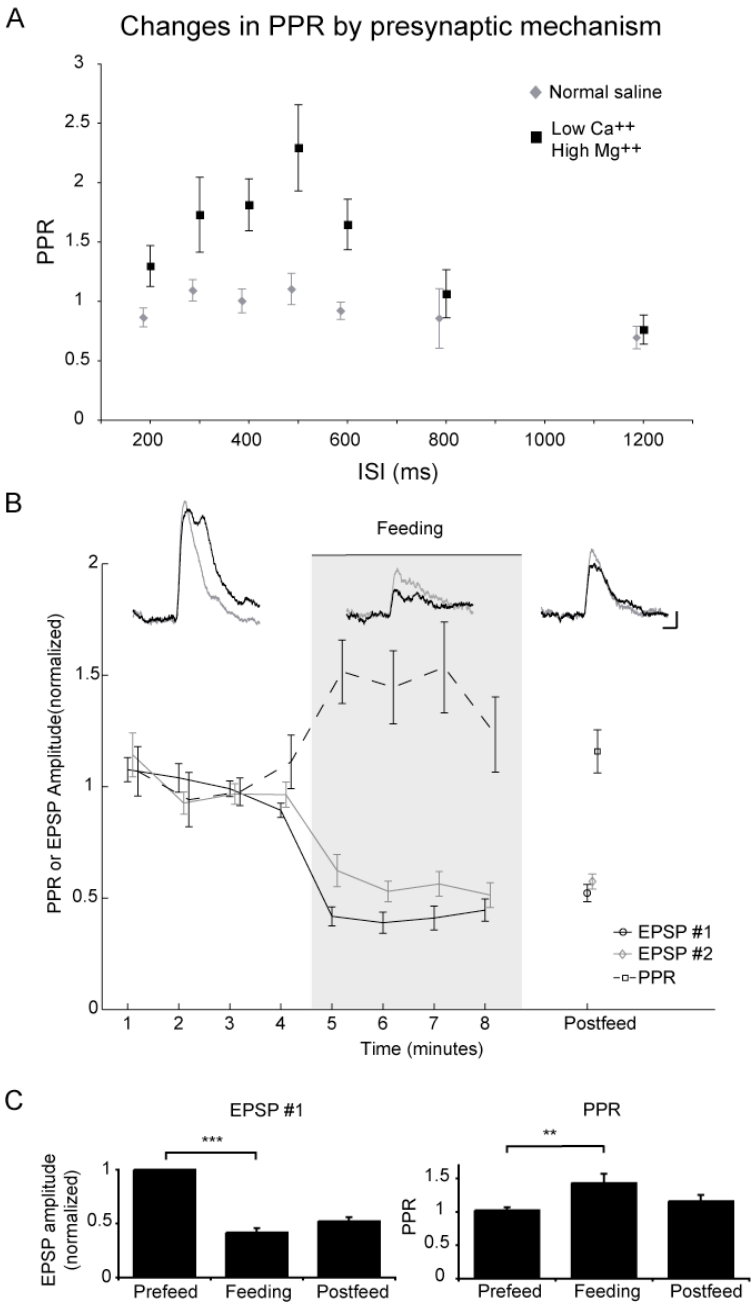
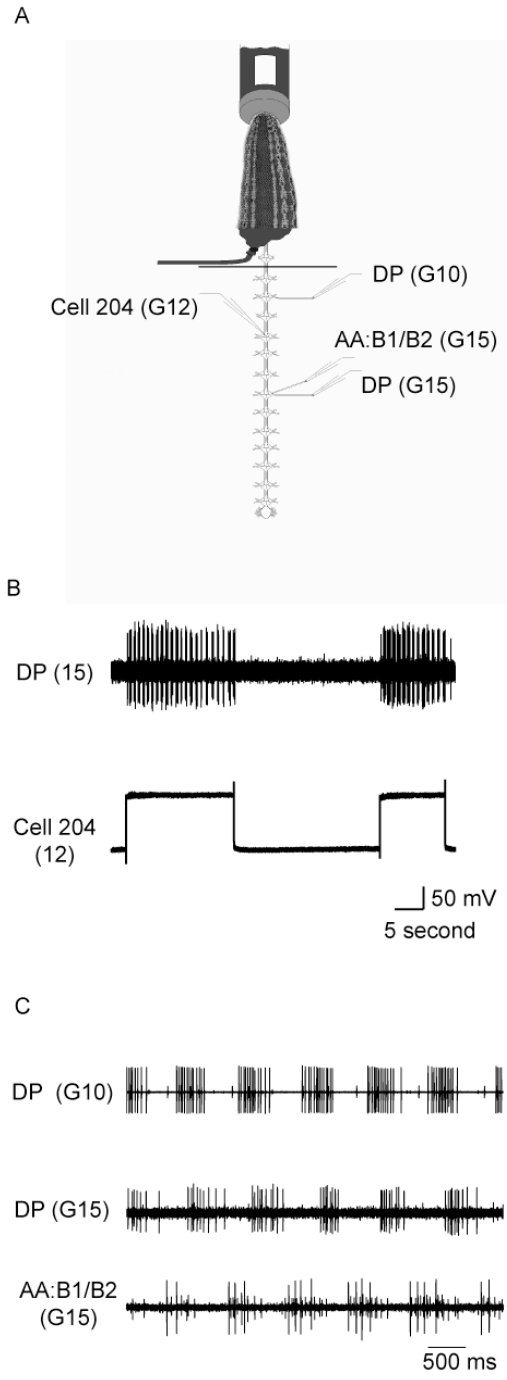

A
Changes in PPR by presynaptic mechanism
Normal saline
Low Ca++
High Mg++
PPR
ISI (ms)
B
Feeding
PPR or EPSP Amplitude(normalized)
EPSP #1
EPSP #2
PPR
Time (minutes)
Postfeed
C
EPSP #1
EPSP amplitude (normalized)
***
Prefeed
Feeding
Postfeed
PPR
**
Prefeed
Feeding
Postfeed

Fig. 2.7: Stimulation of swim gating neurons can elicit bouts of swimming during feeding. **A.** A schematic diagram of semi-intact feeding preparation showing the sites of intracellular stimulation and extracellular recordings. Leeches were fed on warmed bovine serum. Cell 204 was impaled in one ganglion between ganglia 11 through 14. DP nerve recordings were made anterior and posterior to the impaled cell 204 to assure that swimming propagated throughout the CNS. DP nerves contain the axon of a dorsal excitor motorneuron and reveal the dorsal contraction phase of each swim cycle, while the AA:B1/B2 nerve contains the axons of a ventral excitatory motor neurons and indicate the ventral contraction phase of each swim cycle. **B.** Depolarization of cell 204 with 2 nA results in swim-like bursting of the DP nerve recorded in ganglion 15. Traces were recorded while the anterior end of the animal was feeding from the serum tube. Four out of eight animals stimulated in this manner showed swimming activity during feeding. **C.** Sample traces of three nerves taken during a swimming bout initiated by stimulation of cell 204 during feeding. Cell 204 was stimulated in ganglion 12 as shown in panel A. DP nerves 10 and 15 show an anterior to posterior propagation of the dorsal contractions down the nerve cord of the animal. The antiphase spike bursts seen between DP(G15) and AA:B1/B2(G15) reveal the alternation between dorsal and ventral contractions seen within a segment in intact swimming leeches.


A
DP (G10)
Cell 204 (G12)
AA:B1/B2 (G15)
DP (G15)
B
DP (15)
Cell 204
(12)
50 mV
5 second
C
DP (G10)
DP (G15)
AA:B1/B2
(G15)
500 ms

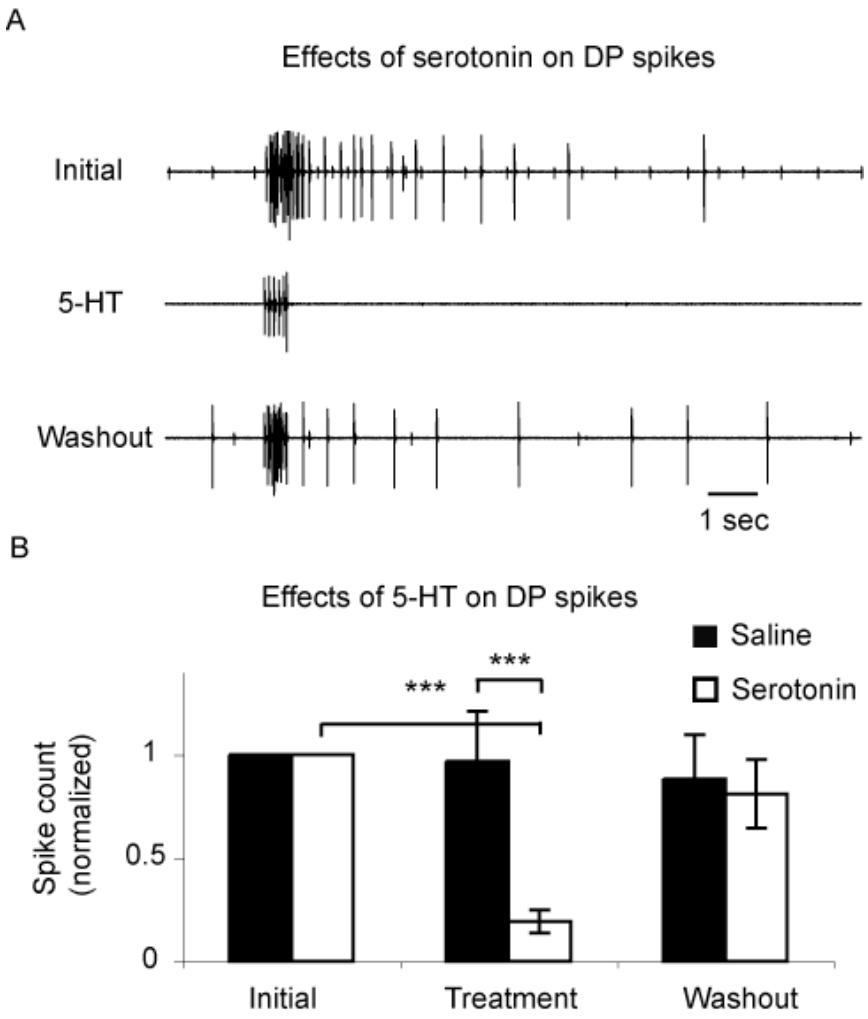


Fig. 2.8: Serotonin mimics feeding by decreasing the motor response to a P cell spike burst. **A.** A train of five spikes was elicited in the P cell and cause a local bend like response in the ipsilateral DP nerve. Bath application of serotonin to the saline reduces the amount of motor activity generated. **B.** Summary bar graph showing a decrease in DP nerve response in the presence of 50 μM serotonin compared to both pre serotonin application and compared to control ganglia. Serotonin application, n = 12. Saline control, n = 4.

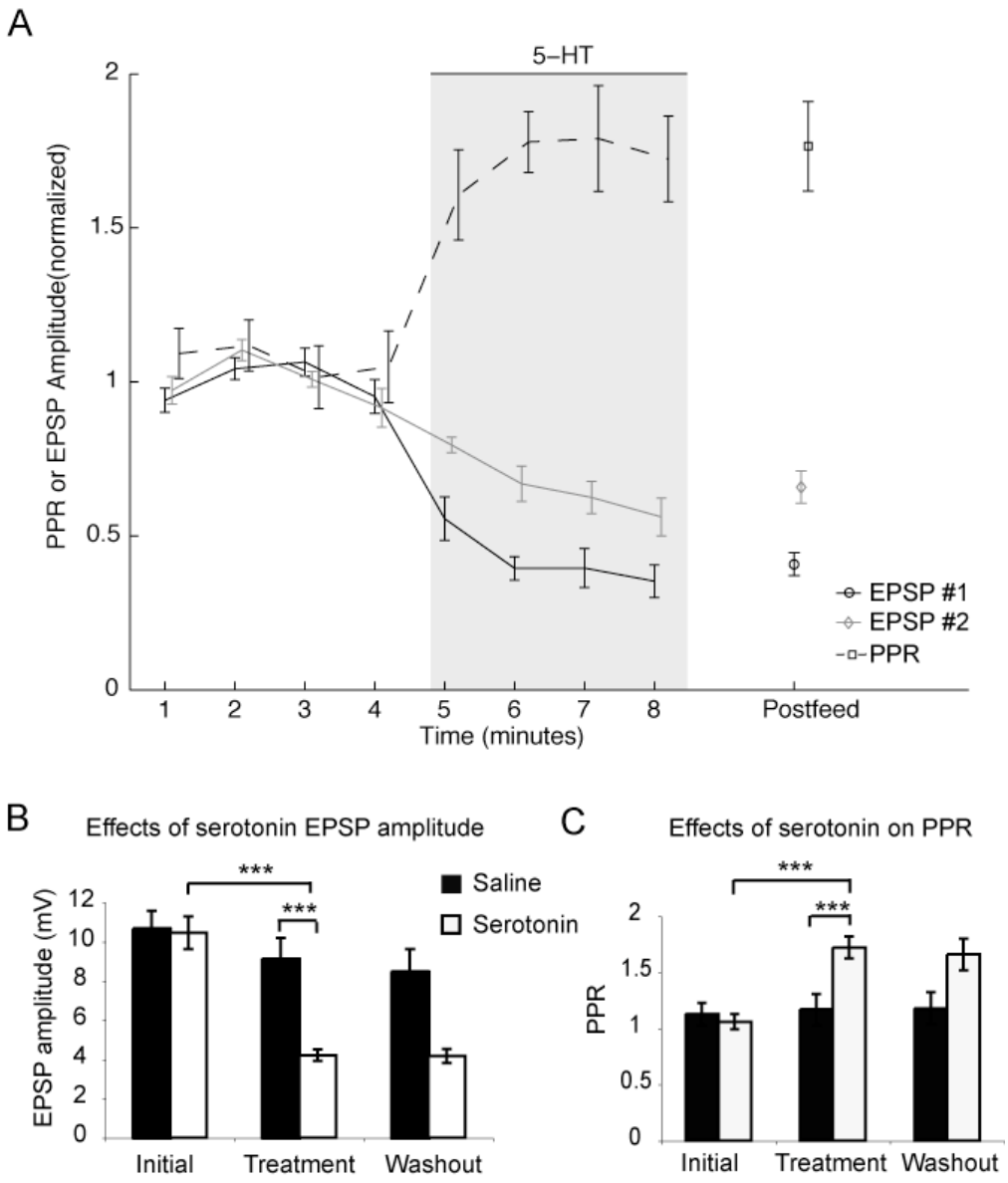


Fig. 2.9: Serotonin decreases the amplitudes of EPSPs between P cells and the AP neurons. **A.** EPSP amplitudes and paired pulse ratios were measured every minute. Application of serotonin (grey box) results in a decrease in EPSP amplitude (first EPSP drawn in black and second EPSP drawn in grey) and an increase in PPR (dashed black line). **B.** Summary plot comparing the EPSP amplitudes of ganglia exposed to serotonin versus unexposed control ganglia. **C.** Summary plot comparing the rise in PPR measured in ganglia exposed to serotonin versus the PPR of control ganglia. N = 8.

3. Distention induced suppression of swimming in the leech

Abstract

The response of an animal to a stimulus can vary with many factors such as age, hormonal state, or experience. The state of an animal can, therefore, be probed by determining how it chooses to respond to a particular sensory stimulus. Part of this decision-making process must involve the suppression of behaviors that are inappropriate or incompatible with the animal's current behavioral state. Swimming behavior in the leech is incompatible with both feeding and the consequent sated state. In this paper, we show that the distention resulting from feeding inhibits leech swimming. Distention decreases the number of swim cycles in response to electrical stimulation and increases the period of swimming. Using semi-intact preparations, we show that this inhibition has a rapid onset (~1 second) and is readily reversible. Distention of the leech gut terminates on-going swim episodes suggesting that the inhibition is targeting the maintenance of swimming rather than its initiation. Through surgical manipulations, we demonstrate that the stretch receptors mediating the distention-induced suppression of swimming are likely to be located in the body wall of the leech as opposed to the CNS itself or the gut lining of the animal. Together with previous results that showed that ingestion during feeding inhibits the initiation of swimming in the leech, these data show that leeches rely on distributed decision-making networks to ensure that an appropriate behavior is expressed at the appropriate time.

Introduction

Animals must perform appropriate behaviors at appropriate times to survive. Different brain regions are often used to perform specific aspects of a decision. For instance, primates make decisions about *when* to make visual saccades in an area of the superior colliculus that is distinct from the area that decides *where* to look (Glimcher and Sparks, 1992; Glimcher, 2001): microstimulation of the superior colliculus selectively influences "where to look", while leaving "when to look" unmodified. Another example is that monkeys store knowledge about reward probability in the lateral intraparietal area (LIP) of the cerebral cortex, whereas information about the value of a reward (i.e., whether it is good or bad, weak or strong) has been found in the basal ganglia (Schultz, 1998; Platt and Glimcher, 1999; Glimcher, 2003).

To make a successful decision, an animal must not only select the appropriate behavior but it must also suppress competing behaviors. The simple nervous systems of invertebrates make them particularly useful for dissecting these types of decision-making processes and determining how neuronal circuits accomplish such behavioral choices. Studies on well-characterized invertebrate circuits have found different mechanisms for suppressing competing behaviors (Kristan and Gillette, 2007), including inhibition between central pattern generators (Jing and Gillette, 1995) and network reconfiguration (Shaw and Kristan, 1997; Jing and Gillette, 1999; Popescu and Frost, 2002).

In the leech, feeding abolishes all mechanically elicited behaviors including swimming (Misell et al., 1998). As leeches begin to feed, descending pathways from

the anterior brain inhibit the release of transmitter from the presynaptic terminals of the pressure mechanosensory neurons (P cells) within the segmental ganglia of the nerve cord, thereby preventing the initiation of behaviors by sensory gating (Chapter 2, this thesis). This presynaptic inhibition of sensory input explains why swimming fails to occur during ingestion but does not address why swimming remains inhibited for at least tens of minutes after feeding is completed (Misell et al., 1998). We investigated later ingestive stages to find additional mechanisms by which feeding accomplishes long-lasting suppression of swimming in leeches.

A hungry medicinal leech can increase its body mass by more than ten-fold (Lent and Dickinson, 1984; Lent et al., 1988), a weight gain that changes the leech's behavior for many weeks. Distending a leech by injecting it with saline terminates feeding (Lent and Dickinson, 1987) and makes it less likely to swim (Groome et al., 1993), presumably by activating stretch receptors. Peripheral stretch and mechanoreceptors are important for coordinating and tuning locomotory responses in a range of animals including *Drosophila* (Song et al., 2007), *C. elegans* (Li et al., 2006), leeches (Cang and Friesen, 2000; Yu and Friesen, 2004), and mammals (Pearson, 2008). In the swimming leech, the stretch receptors are activated out of phase, with those in the dorsal longitudinal muscles activated by the ventral contraction phase of each swim cycle and the ventral stretch receptors activated during dorsal contraction (Yu and Friesen, 2004). We show that feeding produces a hyperextension of the dorsal and ventral longitudinal muscles simultaneously, and propose that the same stretch receptors that alternate to help shape swimming movements inhibit the leech swim circuit when activated simultaneously. These

finding indicate that the inhibition from distention uses both different sensory input and targets different circuitry from the previously described inhibition during the early stage of food ingestion (Chapter 2, this thesis).

Methods

Stimulation protocols: To stimulate the skin of leeches, we delivered trains of electric shocks with a custom hand-held electrode touched to the skin. The terminals of the electrode were 0.008" diameter sliver wire coated with teflon except at the tip, and separated by approximately 1 mm. Shocks were 1 ms trains of 8V pulses at 10Hz delivered through a Grass S88 Stimulator with a Grass Stimulus Isolation Unit (SIU). We stimulated DP nerves with the same protocol except that the pulses were 2-3 volts, delivered through extracellular electrodes.

Severed nerve cord preparations: We first weighed each leech to be able to monitor their body mass increase. We then anesthetized the leech by submerging it in crushed ice made from leech saline. We then made an incision the size of one annulus between segments 4 and 5 through which we severed the nerve cord. We then sutured the incision closed using silk thread. Finally, we trimmed the edges of the caudal sucker using micro dissection scissors to prevent animals from attaching it to the surfaces of the recording chambers; attaching the rear sucker tended to inhibit swimming. To feed the leeches, we used fresh bovine serum (Animal Technologies,

Tyler, Texas) from a 15ml Falcon® tube cut at one end and sealed with sheep intestine (purchased at local delicatessins as sausage casing). We monitored the volume of blood consumed per minute using the markings on the Falcon® tube. Following feeding bouts all leeches were again weighed and their percent body mass increase was recorded. We lanced some leeches through the body wall and gut wall in the mid-region of the leech using a surgical blade (No. 11) and reweighed them after monitoring their swim responses to stimulation. For severed nerve cord preparations, we calculated the mass during feeding (Body Mass (% initial)) as:

$$\text{Body Mass (\% initial)} = \frac{\text{volume decrease in tube} \times \text{blood density} \times 100}{\text{initial mass}} + 100$$

The density of blood used was 1.05 g/ml.

Semi-intact distention preparation (Passive Distention): We anesthetized the leeches using ice-cold leech saline and pinned them through both suckers dorsal-side-up. Through an incision from the head down to ganglion 4, we severed the nerve cord between ganglia 1 and 2 and dissected the DP nerves of ganglia 3 and 4 for extracellular recordings. We then cut the body from ganglion 4 to ganglion 6, leaving the gut intact. We dissected the exposed part of the gut free from the body wall. We also dissected the exposed part of the nerve cord away from the body. At this point, we weighed the preparation as a measure of the initial body mass. We then pinned the dissected leech in the denervated body wall in segments 4 and 5 and cannulated the

gut with PE-100 tubing that was flared on the end by heating it with a soldering iron. We cut the PE tubing short and glued a long thin section of soft silicone tubing to it that we perforated with a scalpel blade. We cut the piece of silicone tubing so that it stopped at the constriction at the intestine (in segment 15). We tied the dissected piece of gut to the flared portion of the PE-100 tubing using No. 6 surgical silk. We perforated the silicone tubing to allow the cannula to add and withdraw fluid into the gut rapidly to the volumes seen in hungry and satiated states. As in the active feeding preparations, we trimmed the caudal sucker to prevent the animals its attachment to chamber surfaces. We attached a syringe to the cannula to control and measure distention levels. Passive distention preparations, we calculated the percent initial mass (Body Mass (% initial)) as:

$$\text{Body Mass (\% initial)} = \frac{\text{volume ejected from syringe x density of saline x 100}}{\text{initial mass}} + 100$$

The density of the saline used was 1.00 g/ml.

Gut-removed preparation (Passive Distention (GR)): To gain access to the gut, we turned a leech inside-out, by first anesthetizing it in ice-cold saline, pinning it out ventral-side-up, and decapitating it between segments 3 and 4. We then moved it into 5°C leech saline containing 8% ethanol for 10 minutes to completely relax its muscles. To turn the leech inside-out, we used a 200µl pipetteman tip to force its posterior

sucker into its body cavity about half-way to the opening at the anterior end. We then removed the pipette tip and used a fire-polished glass capillary tube (O.D. 1.0 mm) to complete the inversion. Once inverted, the leech was placed into a dissection tray containing ice-cold saline without ethanol. (One preparation was placed in saline containing 50 μm serotonin, which improved the performance and the life span of the preparation.) To remove the gut, we secured the leech by pinning several latex strips across it; we also left the glass capillary tube inside the animal, which helped to secure it. The entire gut of the animal was then removed by starting at the exposed CNS and working outward around the circumference of the animal. Great care was taken to follow the nerves from the CNS and to avoid damaging them. We removed as much of the intestine as possible and sutured it closed to prevent saline from leaking out the anus. We then turned the leech back to its original (outside-out) state using the glass capillary tube.

The eviscerated leech was then cannulated using PE-100 tubing with a heat-flared tip and perforated silicon tubing extension as described above, except that the body (rather than the gut) was tied tightly to the cannula and the end of the PE tubing was attached to a syringe filled with saline to inject and remove saline. Serotonin (50 μM) was sometimes added to the injection saline; it prolonged the life of the preparation but did not change the effects of distention on swimming. The posterior sucker of the leech was incapacitated by applying a drop of super glue into the center of the sucker. We allowed the leech to recover for an hour or until they became active on their own. We transferred the leech to a 400 ml beaker and held it suspended in

leech saline by the cannulation tubing; we stimulated the animal and quantified its swimming performance. The calculations of percent initial body mass for these preparations with were the same as for passive distention semi-intact preparations (see above).

Isolated nerve cord preparation: We first imaged leeches to determine the change in body length during resting, swimming, crawling, and after feeding. We then selected a separate, sized matched group of leeches and recorded their maximally contracted state during crawling. These leeches were then dissected and their nerve cords were removed. The isolated nerve cord preparation was performed as previously described (Kristan and Calabrese, 1976). Nerve cords consisting of ganglia 2 through the tail brain were removed from the leech and placed into a recording chamber containing 50 μm serotonin to promote swimming (Willard, 1981; Hashemzadeh-Gargari and Friesen, 1989). We securely pinned down ganglion 10 and used extracellular electrodes to stimulate and record from its DP nerve. Latex strips were pinned down across the nerve cord between ganglion 10 and adjacent ganglia to secure the nerve cord. The connective anterior to ganglion 2 and the tail brain were loosely pinned and repinned throughout the experiment to vary the length of the nerve cord.

Regressions and statistics: For each regression analysis, the data from individual animals were first averaged between 100 to 150 percent initial body mass, 150 to 200 percent initial body mass, and every 100 percent increase in body mass thereafter. Regression analyses were performed on custom Matlab software and

verified using Microsoft Excel. We tested for differences in slope and elevation between active feeding preparations, passive distention preparations, and passive distention preparations with their guts removed according to Zar Biostatistical Analysis. A logarithmic fit of the data was used when its R^2 value was higher than the R^2 value obtained using a linear fit. In experiments varying the nerve cord length of animals, the cord lengths were normalized to each animal's fully contracted length prior to dissection. Cord lengths were then binned and averaged within an animal at each 50% change in cord length before comparisons between animals were made.

Results

Distention inhibits swimming

Leeches will not respond to stimulation during feeding (Misell et al., 1998) because descending inhibition targets the tactile sensory terminals of pressure mechanosensory neurons (Chapter 2). To remove this inhibition we severed the connectives of the nerve cords of otherwise intact leeches between ganglia 4 and 5 (Figure 3.1A), thus allowing the anterior end of the animal to feed normally while the posterior end of the animal produced normal swimming behavior. Leeches do not require the anterior brain to generate normal swimming behavior; in fact, removal of this brain leads to more reliable swimming behavior (Brodfuehrer and Friesen, 1986). We fed the leeches from an inverted Falcon® tube fitted with sheep intestine (sausage casing). We used the calibrations on the Falcon® tube to monitor the quantity of blood

consumed by leeches each minute. To initiate swimming, we stimulated the rear end of the leeches electrically once per minute (Kristan et al., 1982) and counted the number of swim cycles produced during the next minute.

Leeches were only stimulated while feeding, and the duration of feeding bouts varied between animals. Thus the number of leeches studied decreased as time increases in our experiments (Figure 3.1B, upper plot). During a feeding bout, the number of swim cycles elicited in response electrical stimulation decreased (Figure 3.1B, black trace) as the mass of the feeding animal increased with time (grey trace; N = 15 leeches). To rule out the possibility that habituation or exhaustion caused this decrease in swim cycles over the 60-minute interval, we exposed 4 animals to the same surgical and stimulation procedures but did not allow them to feed. The number of swim cycles in these animals decreased slightly over the hour (Figure 3.1B, black dashed line) but was significantly higher than the number of cycles produced by feeding animals. This result shows that habituation and run-down accounted for only a minor portion of the suppression of swimming. To show the correlation between increases in body mass and the number of swim cycles, we replotted these two measurements against one another (Figure 3.1C). The black line indicates the best-fit line ($R^2 = 0.59$, $P = 7.5 \times 10^{-30}$, N = 15 leeches, 144 trials). All individual leeches showed a robust decease in elicited swim cycles with increasing body mass (Supplementary, Figure 3.7), so that much of the variability in Figure 3.1C was due to between-animal variation.

To determine whether the increased mass in the body of the animal caused the decrease in the number of swim cycles produced, we performed two additional

experiments. First, we fed five leeches to satiation, and then lanced them (by cutting through the body wall and into the gut using a scalpel) to drain them of much of their ingested blood meal. The lancing decreased their body mass and returned the number of stimulus-induced swim cycles to slightly above the levels previously found for the pre-lancing body weight (Figure 3.1C, + symbols). Second, we developed a semi-intact preparation to passively inflate the gut with fluid without the leech actively feeding (Figure 3.2A). We removed the body wall anterior to ganglion 6, leaving only the nervous system. We left the DP nerves on either ganglion 3 or 4 intact and used them to monitor swim episodes. We dissected the gut tissue in segments 5 and 6 free of the body wall and inserted through it a perforated cannula into the gut of the intact body. We tied off the gut around the cannula and secured the cannula to the body wall. We attached a syringe filled with saline to the end of the cannula, which we used to add and remove measured amounts of saline to the gut repeatedly (Figure 3.2B). We used the nerve recordings (Figure 3.2C) to monitor the number of swim cycles produced by a single stimulus train (every swim burst in the DP nerve was accompanied by a swim cycle in the intact posterior region of the leech). Increases in distention levels in passively distended semi-intact leeches caused a decrease in the number of swim cycles in response to stimulation (Figure 3.2D, black dots, black line is best fit line, $R^2 = 0.63$, $p = 4.1 \times 10^{-5}$, N = 3 leeches, 19 trials). The regression line for these data (Figure 3.2D, black line) are not significantly different from the regression line found in actively feeding leeches (Figure 3.2D, dashed grey line).

Distention also affected the period of swim cycles. The period of swimming was calculated from the bursts of cell 3 action potentials in the DP nerve. The swim

cycle period typically increases (i.e., swimming slows down) as the swim episode progresses in fully intact leeches as well as in semi-intact preparations and isolated nerve cords (Kristan and Calabrese, 1976), so we needed to compare similar parts of swim episodes across different conditions. Because the number of swim cycles per episode was small in fully distended leeches, we used the burst period during only the first 5 seconds of each trial. We found that increases in distention significantly increased the swim period (Figure 3.2E, $R^2 = 0.61$, $P = 1.2*10^{-4}$, N = 3 leeches, 18 trials).

Because we could also control the rate of distention in the semi-intact preparation, we determined how quickly distention turned off the swim central pattern generator. We initiated swimming in semi-intact leeches while the gut was empty, then distended the gut rapidly (Figure 3.3). From the previous experiments, we knew that swim episodes in non-distended leeches could last up to one minute (Figure 3.1B, unfed controls). When strongly distended (to about 800% of initial body volume), however, swimming terminated within 3 seconds (Figure 3.3A), and most of this time was required to pass the fluid into the animal without rupturing the gut. Often, swimming would spontaneously resume within several seconds after the release of distention. The effect of distention was robust, and did not habituate even after many repetitions of distention and release of distention (Figure 3.3B). Averaging across four animals (Figure 3.3C) showed that the onset of the decrease in swimming in distended trials versus non-distended trials was within 1 second of distention onset and all distended trials across all leeches had stopped swimming by 3 seconds ($p < 0.01$ at 1 second and $p < 0.001$ between 2 to 6 seconds, N = 4).

Location of distention receptors

To determine the site of the distention receptors, we considered three Sites: within the CNS, in the lining of the gut itself, or in the body wall enclosing the gut. We first tested whether the stretch receptors are located within the CNS. The leech nervous system is highly elastic and stretches as the animal elongates during behaviors such as swimming and crawling (Wilson et al., 1996). To determine how much the nerve cord is stretched as the animal expands during feeding, we video-taped leeches as they crawled, rested, swam, and after they had fed to see how much change in the length the leech and nervous system typically undergo. We can approximate the length of the nerve cord in intact leeches by measuring the length of the animal from the middle of the anterior sucker to the middle of the posterior sucker, the locations of the head and tail brains. We found that the length of the leech CNS undergoes large changes as the leech performs various behaviors (Figure 3.4A). Because the shortest length is achieved during the contraction phase of crawling, we normalized all body lengths were normalized to this contracted length. Prior to feeding, the maximum leech achieved was 3 times that of the fully contracted state; this extended length was achieved throughout swimming and during the extension phase of crawling. After feeding to satiation (which, as seen in Figure 3.1B, produces a 1200% increase in body mass), leeches averaged a 3.9 times increase in body length. Hence, to mimic this length change, we stretched the isolated nerve cord to a length at least as long as it would be in these sated animals.

To perform these experiments, we removed the entire CNS, minus the anterior brain (to facilitate swimming in the isolated nervous system), then stimulated the DP nerves in ganglion 10 and recorded from them to monitor swimming (Kristan & Calabrese, 1976). We secured the isolated nerve cord at ganglion 10 and pinned the two ends of the nerve cord at different lengths, then initiated swimming episodes and measured number of swim cycles elicited within 1 minute of stimulation. Varying the cord length over the physiological range had an insignificant effect on the number of swim cycles elicited (Figure 3.4B, $R^2 = 0.13$, $P = 0.12$, $N = 4$ leeches, 19 trials). The dashed vertical lines in Figure 3.4B correspond to the mean cord lengths of intact animals engaged in the contraction phase of crawling, at rest, engaged in the elongation phase of crawling, swimming, or at rest after feeding (data taken from Figure 3.4A). These data show that stretching of the leech CNS does not contribute significantly to swim inhibition by distention.

To determine whether stretch receptors in the wall of the gut sensed distention, we removed the gut from leeches and tested the effect of distention on swimming. To get access to the gut, we removed the anterior end of the leech through segment 4, then turned the leech inside-out by pushing the tail sucker through the leech's internal cavity and out through the severed anterior end. With the animal in this state (Figure 3.5A left), we could readily dissect the entire exposed gut of the animal (Figure 3.5A right). After all gut and intestinal tissue had been removed, the leech was returned to its normal outside-out condition by pushing the tail sucker back through the animal. We then cannulated the cut anterior portion of the leech and tied it to the body wall. After a recovery period of approximately one hour, we stimulated the eviscerated

leeches to swim and counted their swim cycles. Injecting fluid into the body cavities of these animals to distend them caused a decrease in the number of swim cycles (Figure 3.5B). The linear regression for data did not differ in either slope or elevation from data obtained from severed nerve cord preparations, semi-intact preparations, or animals with their gut removed. These data show that receptors in the gut are not necessary for the suppressive effects of distention on the number of swim cycles.

Discussion

We have shown that a critical component of leech feeding behavior, distention, inhibits the leech swim circuitry. This inhibition has a rapid onset and appears to target the maintenance of swimming rather than its initiation. We have also shown that the stretch receptors that mediate this suppression are most likely not located in the CNS of the leech or in the gut lining itself. We therefore conclude that they are most likely located within the body wall musculature of the animal.

Origins and targets of distention induced inhibition

We believe that the stretch receptors responsible for the distention induced suppression of swimming are the same as those originally described by Blackshaw and Thompson (1988), and subsequently shown to be important in shaping leech swimming (Cang and Friesen, 2000; Yu and Friesen, 2004). Unlike the stretch receptors of vertebrates, leech stretch receptors hyperpolarize in response to stretching

of the body wall and depolarize in response to the release of tension. The ventral stretch receptor (VSR) has been shown to interact with motor neurons and interneurons responsible for generating the swim rhythm (Cang et al., 2001). Stretching of the VSR during the dorsal phase of swimming leads to a hyperpolarization of motor neurons and interneurons that are active in the dorsal contraction phase of swimming and a depolarization in neurons that are normally active in the ventral phase of swimming. Although similar studies have not yet been conducted on the dorsal stretch receptor (DSR), it is believed that the DSR has mirrored effects on the leech swim circuit to work in unison with the VSR to provide a more robust rhythm. It is currently unknown how distention and thus simultaneous activation of the VSR and DSR would affect the swimming circuit.

The increase in swim period that we observed during distention has been previously mimicked through current injections into the swim gating neuron, cell 204 (Weeks and Kristan, 1978). The endogenous firing rate of cell 204 is also highly correlated with the period of swimming (Debski and Friesen, 1986). The gating cells provide excitation for the central pattern generator, which in turn drives the motor neurons (Weeks, 1982; Nusbaum et al., 1987). While the interaction between the gating cells and the CPG appears to be an ideal candidate target from distention induced inhibition, it is currently unknown whether distention decreases the excitation from the gating neurons or the ability of CPG cells to respond to such excitation.

Behavioral switching to crawling after feeding

There are a plurality of intrinsic and extrinsic factors that influence the decision to swim including the depth of water surrounding the leech (Esch et al., 2002) , the level of various neuromodulators (Crisp and Mesce, 2003, 2006), and the satiation level of the leech (Misell et al., 1998). After feeding, leeches will choose to crawl away from tactile stimulation as opposed to swimming. The switching from swimming to crawling observed after feeding could be the result of changes to cell 208, a neuron critical in the decision of whether to swim or crawl in response to stimulation (Briggman et al., 2005). Cell 208 is a unique neuron within the swim circuit and also makes unique connections with the VSR. Cell 208 is the only excitatory member of the swim CPG and functions to connect the swim gating neurons to the swim CPG (Weeks, 1982; Nusbaum et al., 1987; Kristan et al., 2005). Cell 208 is active during the dorsal phase of swimming but unlike other cells active in the same phase, cell 208 depolarizes in response to ventral stretch in contrast to other cells, which hyperpolarize (Cang et al., 2001). Furthermore, the connections between the VSR and other CPG neurons appear to be polysynaptic and the interactions are only seen when manipulations of the VSR are made during fictive swimming. However, cell 208 depolarizes in response to VSR hyperpolarization even in the quiescent state which may be the result of a direct connection between the stretch receptor and cell 208 (Cang et al., 2001). Hyperpolarization of the ventral stretch receptors, as might occur during distention, is sufficient to depolarize cell 208 and depolarization of cell 208 biases leeches to crawl in response to DP stimulation (Briggman et al., 2005). Thus it is possible that distention not only inhibits swimming by inhibiting swim related neurons, but also promotes crawling by targeting the decision-making neuron

208. It will be necessary to determine the simultaneous effect of dorsal and ventral stretch receptor activation on cell 208.

The inhibition of swimming during feeding is distributed and modularized

Feeding in leeches appears to inhibit the swim circuit in both a distributed and modularized manner (Figure 3.6). The decision to stop swimming is distributed in that it most likely arises from two distinct sources, one that generates ingestion, and the other that is activated by distension. We have shown previously that ingestion activates a descending pathway that targets the pressure mechanosensory neurons (P cells) of the leech. This descending inhibition decreases the probability of release measured at presynaptic release sites of the P cells. Figure 3.1 in this current study further illustrates this point by showing that severing the nerve cord is sufficient to relieve posterior segments of this inhibition and allow swimming to occur simultaneously with feeding. The inhibition of the leech swim circuit by distention appears to originate from a different source, most likely the stretch receptors located within the body wall musculature of the animal. Unlike ingestion, distention targets components of the swim circuit downstream of the sensory neurons.

Because ingestion and distention have two distinct sources and targets for their inhibition we can think of them as unique decision-making modules, or we can describe the decision making process as modular. Why have two different modules to inhibit swimming during feeding? Are the two mechanisms redundant or do they play different roles in suppressing behavior? The two mechanisms do not appear to be

redundant but are better described as complementary. Ingestion prevents the initiation of swimming and distention inhibits the maintenance of swimming. More importantly however, the effects of ingestion are to broadly inhibit all mechanically elicited behaviors while distention appears to leave some behaviors intact while suppressing others such as swimming. By segregating different components of the decision-making process into separate modules, the leech can recruit either strategy independent of the other. For example, during the ingestion phase of feeding, it is important to suppress all mechanically elicited behaviors that may compete and interfere with ingestion. Thus direct inhibition of the mechanoreceptors is an ideal strategy to prevent competing behaviors from being expressed. Once feeding is terminated, the leech faces a new challenge. It must now regain the ability to respond adequately to tactile stimulation but must still bias its actions away from behaviors that are mutually exclusive with being in the distended state, such as effective swimming. The distention acquired during feeding can now serve to suppress swimming in the post feeding state and bias the leech towards crawling.

Distributed and modular decision-making systems may be a common theme of the nervous systems across animal phylogenies. Complex decisions often contain several independent steps and the underlying circuitry governing those steps may be separable. In the primate visual saccade system, different brain structures are used to determine of where and when to saccade (Glimcher and Sparks, 1992), determine the probability and value of a reward (Platt and Glimcher, 1999; Glimcher, 2003), and updating knowledge of prior probabilities and rewards (Schultz, 2000; Schultz and Dickinson, 2000). By utilizing distributed and modularized decision-making circuits,

animals may combine or express different decision-making modules as the task or environment around them changes.

Acknowledgements

The work contained within this chapter is currently being prepared for future publication. The data in Figure 3.1 was collected in collaboration with Natalie Ruiz and used with that co-author's permission.

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Fig. 3.1: Active feeding increases the body mass in leeches and decreases the number of swim cycles. **A.** Schematic diagram of a leech used in the active feeding experiments. Through a small incision in the ventral body wall exposing the connectives between ganglia 4 and 5, we cut the nerve cord to remove the descending neural inhibition of swimming during feeding. We trimmed the tail sucker to prevent its attachment to the substrate, which tends to inhibit swimming. **B.** The relationship between the length of a feeding bout, number of swim cycles elicited by stimulation, and the increase in body mass. Leeches were only stimulated while feeding, and the duration of feeding bouts varied between animals. Thus the number of leeches studied decreases as time increases (upper plot). The black line shows the decrease in swim cycles observed within 1 minute after stimulation as the feeding progresses. Error bars in this figure and all subsequent figures represent SEM. The solid grey line indicates a progressive increase in body mass with duration of feeding, measured as percent of initial body mass. The dashed black line shows the average number of swim cycles for animals that had their nerve cords severed but were not fed (N = 4). **C.** Log plot of the decrease in number of swim cycles elicited by stimulation as a function of increase in body mass (N = 15 leeches). Data are binned for each leech between 100 to 150, 150 to 200, and every 100 percent increase in initial body mass thereafter. The + symbols indicate data for the 5 leeches that were lanced following feeding. The line shows the linear regression (N = 15 leeches, 144 trials, $R^2 = 0.59$, $p = 7.5 \times 10^{-30)}$.

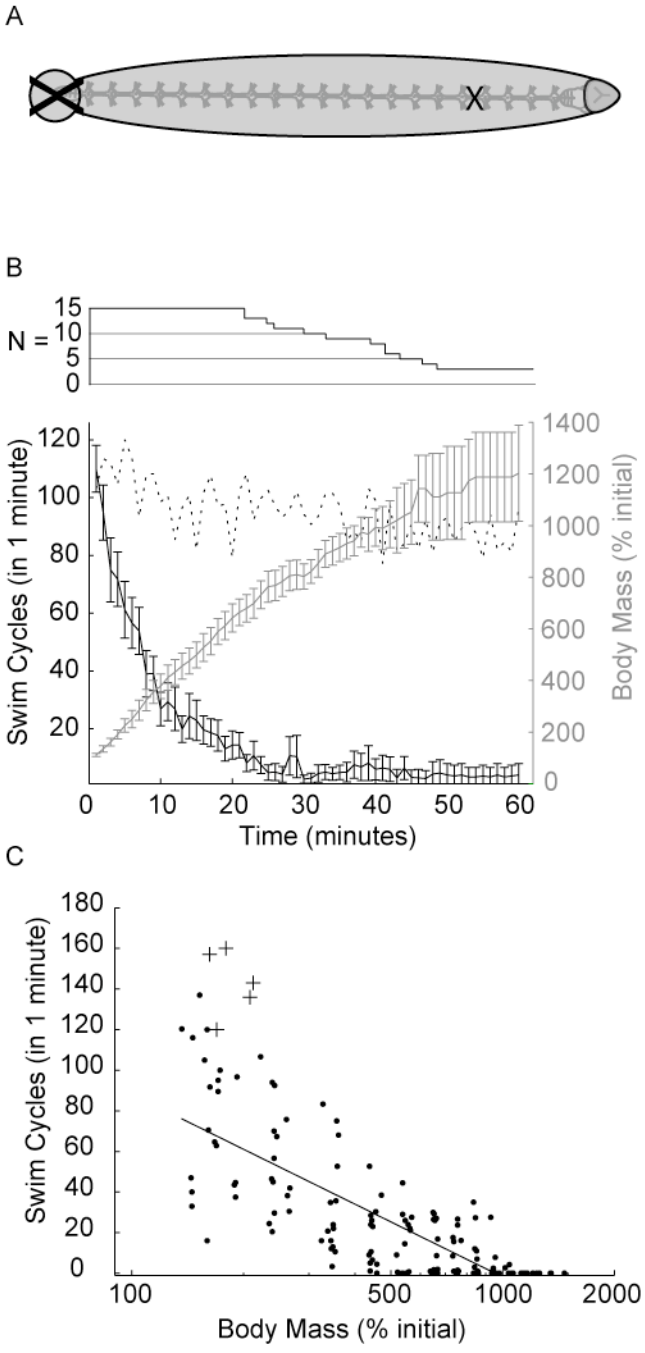

A
X
B
N =
15
10
5
0
Swim Cycles (in 1 minute)
120
100
80
60
40
20
Body Mass (% initial)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (minutes)
C
Swim Cycles (in 1 minute)
180
160
140
120
100
80
60
40
20
0
100
500
1000
2000
Body Mass (% initial)

Fig. 3.2: Passive distention reduces the number of swim cycles in response to stimulation in a semi-intact preparation. **A.** Schematic diagram of the semi-intact preparation used for these experiments. We severed the nerve cord between ganglia 2 and 3 and made extracellular recordings from the DP nerves of ganglion 3 or 4. We then inserted a long perforated tube into the gut of the intact part of the animal and attached it to the open end of the gut with silk sutures (see methods). Saline solution was injected via a syringe to monitor the amount of distention in the preparation. **B.** Sample data from one semi-intact preparation. We performed 15 trials with 3 minutes between trials. Distention levels were calculated from the amount of saline solution injected into the animal. Experiments consisted of several trials were distention values were increased to a maximum and then decreased to a minimum level of distention in a stepwise manner. The O symbol is data from the trace below. **C**. Sample trace of a DP nerve recording showing the motor neuron bursts that define swimming. Between each pair of bursts, the intact portion of the leech swam one complete cycle. At zero time, we stimulated the body wall electrically, as indicated by the large stimulus artifact. Motor activity that precedes the stimulus is from contact made from the stimulating electrode onto the body wall prior to stimulation. We counted all swim bursts within one minute after stimulation. Insert shows an expanded view of the swim bursts between 20 and 25 seconds within the swim episode. **D.** The effect of induced distention on the number of swim cycles observed within 1 minute of stimulation. The X axis is a logarithmic scale because this relationship appeared to be exponential. The black line is the linear regression for these data points. Data were binned as described in Methods and Figure 1. Total averaged data includes 19 trials from 3 leeches. Dashed grey line shows best fit line from active feeding preparations (Figure 1) for comparison. ($N = 3$ leeches, 19 trials, $R^2 = 0.63$, $p = 4.1 \times 10^{-5}$). **E.** The swim period increases with increased distention. The swim period was measured as the time interval between the median spikes in sequential bursts. Periods were measured only within the first 5 seconds of each trial and averaged. ($N = 3$ leeches, 18 trials, $R^2 = 0.61$, $p = 1.2 \times 10^{-4}$).

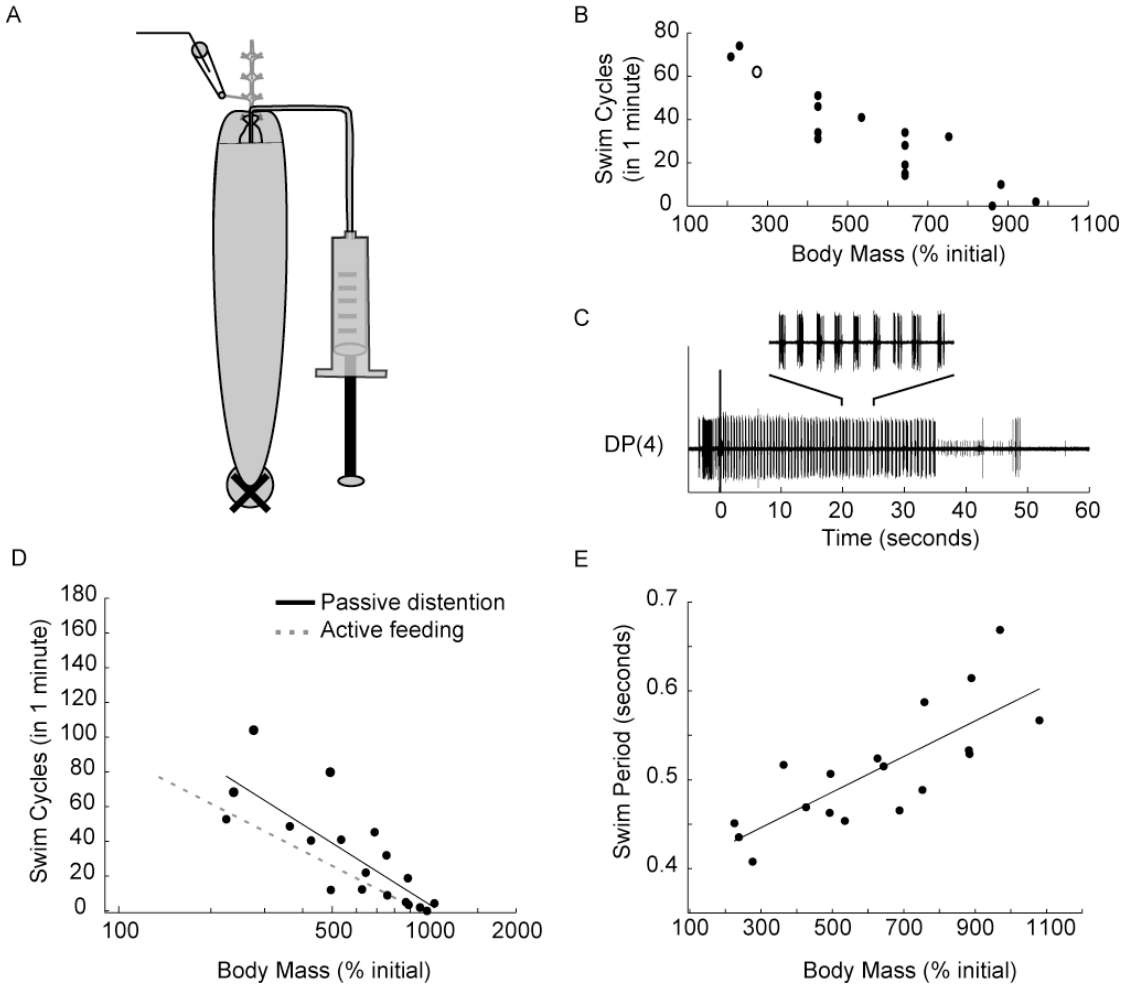

A
B
Swim Cycles (in 1 minute)
Body Mass (% initial)
C
DP(4)
Time (seconds)
D
Passive distention
Active feeding
Swim Cycles (in 1 minute)
Body Mass (% initial)
E
Swim Period (seconds)
Body Mass (% initial)

Fig. 3.3: Distention of the leech gut inhibits ongoing swim episodes. **A.** Nerve recording from DP(3) of a spontaneously swimming semi-intact. Once the animal was swimming, we injected saline into the gut cavity rapidly until swimming ceased (grey boxes). The volume of saline injected distended the leeches to approximately 800% of their initial body mass. Once the saline was removed, swimming would spontaneously resume some seconds later. **B.** Sample trials from one animal showing the effects of distention on the length of swim episodes. We delivered electrical stimuli at the onset of the recording. Trials alternated between non-distended controls and trials in which saline was injected in the gut of leeches during swimming. The traces on the left show the duration of swim episodes in which saline was not injected. The traces on the right show that swim episodes terminated after saline was injected. Grey boxes over each trace show the time of distention. In some trials swimming resumed after the distention was relieved. **C.** Summary of the effects of distention on swim episodes. For each time point, the percentage of trials where swimming had not yet ceased was first calculated and averaged within each animal. These data were then averaged across animals and plotted as a function of time relative to the presentation of the distention. Zero time represents the onset of distention for each distention trial (black line) and the same time point relative to the first swim motor burst in the non-distended trials (dashed grey line). Because the duration of the distention varied somewhat between trials and animals, we plotted the average time of distention +/- SEM. Distention produced a rapid suppression of swim bursts that lasted for many seconds after the distention was relieved (ANOVA with Bonferroni post hoc test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, $N = 4$) (the same symbols are used throughout).

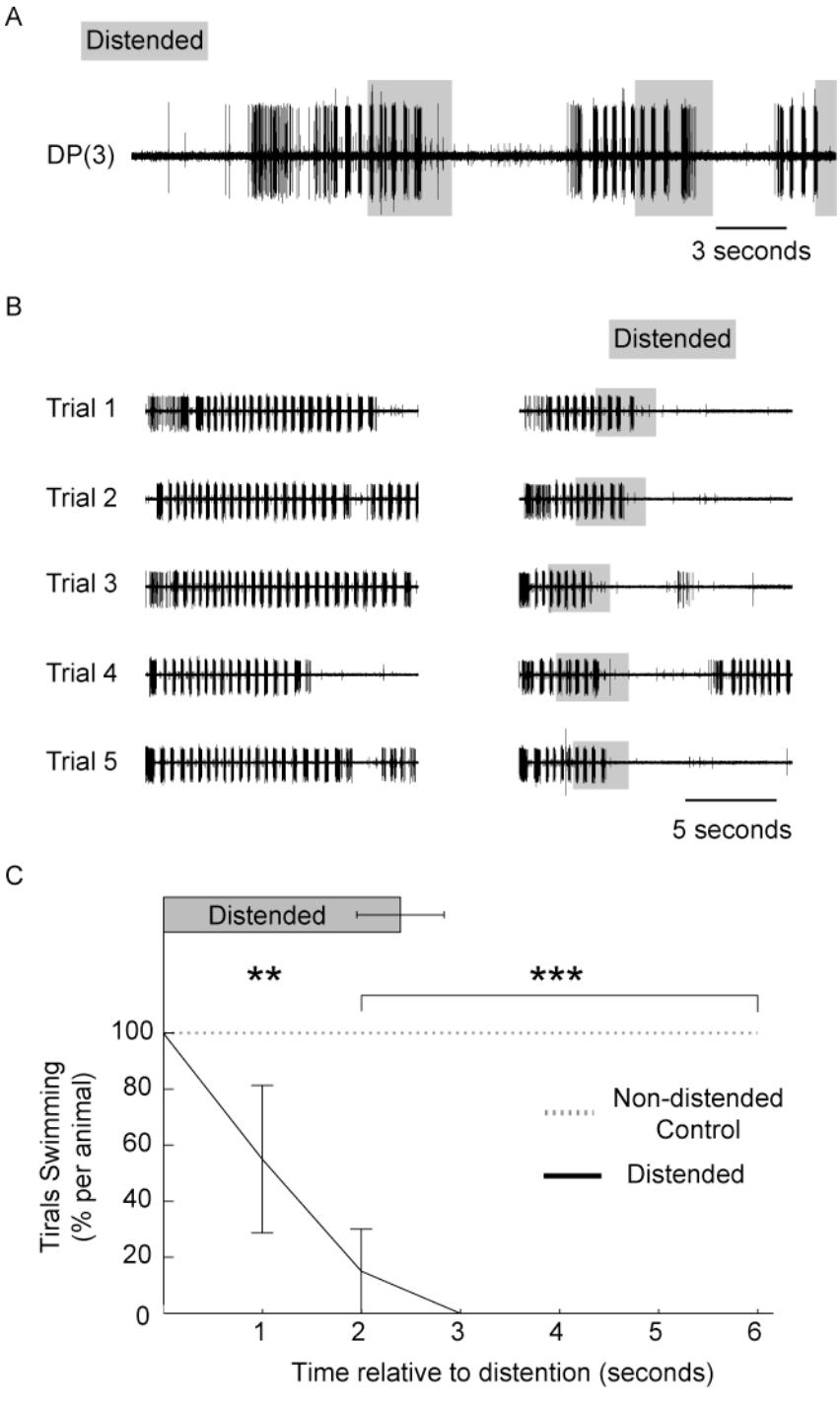
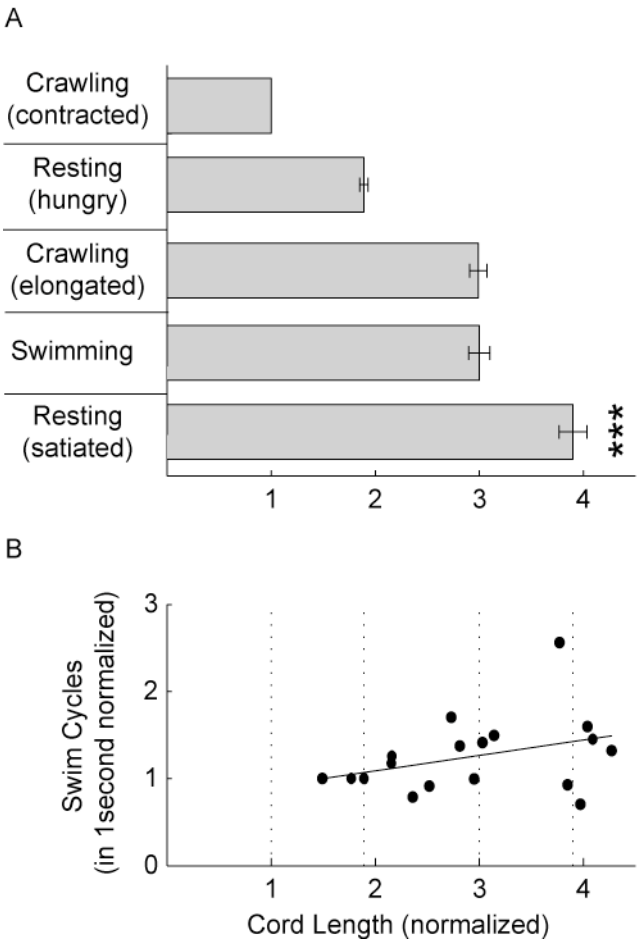

A
Distended
DP(3)
3 seconds
B
Distended
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
5 seconds
C
Distended
**
***
100
80
60
40
20
0
Tirals Swimming
(% per animal)
Non-distended
Control
Distended
1
2
3
4
5
6
Time relative to distention (seconds)

Fig. 3.4: Stretching the leech nerve cord does not affect the swimming rate. **A.** The bars show the relative amount of stretching that occurs when intact leeches perform various behaviors, normalized to the minimum length achieved during the contraction phase of crawling. We measured the maximum and minimum lengths achieved in videotapes of hungry leeches as they swam, crawled (maximum and minimum lengths), and at rest (both hungry and satiated). The lengths of the nerve cords were estimated as the length between the middle of their front sucker and the middle of their posterior sucker (N = 5). The shortest length achieved in unfed animals was during the contraction phase of crawling, and the longest was during the elongation phase. Fully sated leeches were longer--even at rest--than was ever achieved by hungry leeches. (ANOVA with Bonferroni *post hoc* test comparing satiated leeches at rest to all other conditions, $p < 0.001$, N = 4) **B.** Relation between the length of the leech nerve cord and the number of swim cycles elicited by DP nerve stimulation. Data points indicate 19 trials in 4 leeches; $R^2 = 0.13$, $p = 0.12$). Cord lengths were normalized to each intact animal's contracted length. Cord lengths were then binned and averaged at each 50% change in cord length within an animal. Swim cycles elicited within 1 minute of stimulation were normalized within each animal to the number of swim cycles observed in the maximally contracted state. Vertical dashed bars show the mean cord lengths for animals during the five behavioral states shown in A.


A
Crawling (contracted)
Resting (hungry)
Crawling (elongated)
Swimming
Resting (satiated)
***
1
2
3
4
B
Swim Cycles (in 1second normalized)
0
1
2
3
1
2
3
4
Cord Length (normalized)

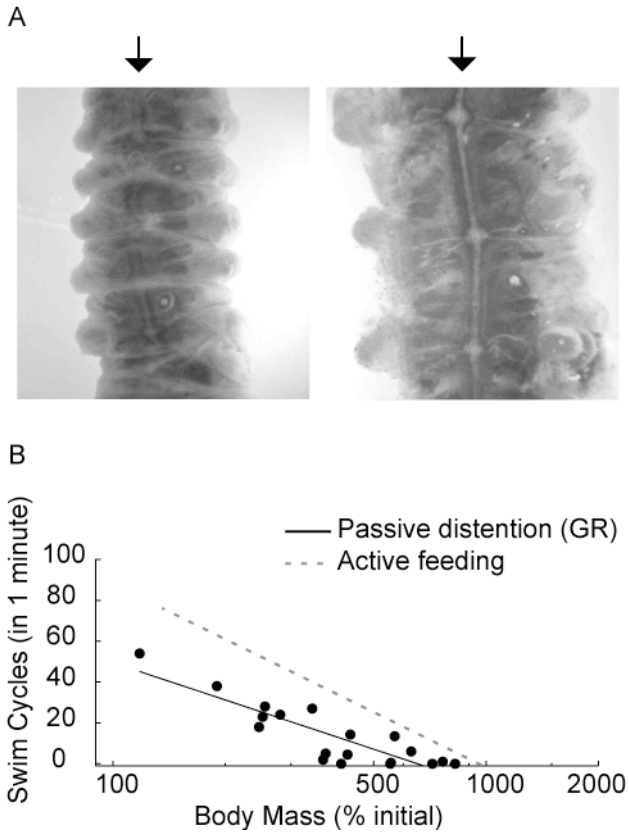


Fig. 3.5: Removing the gut of the leech does not eliminate the distention-induced suppression of swimming. **A (*left*).** Photograph of a leech turned inside-out with the gut intact. The black arrow points to the central nervous system, which is covered by the gut. **A (*right*).** Photo of the same inside-out leech after all the gut tissue has been removed. The CNS (indicated by black arrow) and peripheral nerves are more readily visible. **B.** The effect of induced distention on the number of swim cycles elicited by DP nerve stimulation in eviscerated leeches. The swim cycles in leeches with guts removed (Passive distention (GR)) decreases as percent initial body mass increases (19 data points from 4 leeches; $R^2 = 0.76$, $p = 9.4 \times 10^{-7}$). The solid black line shows the linear regression for the data points shown and the dashed grey line is the regression line for data obtained from intact leeches (Figure 1C). These two lines do not differ in either their slopes (P = 0.69) or their elevations (P = 0.97).

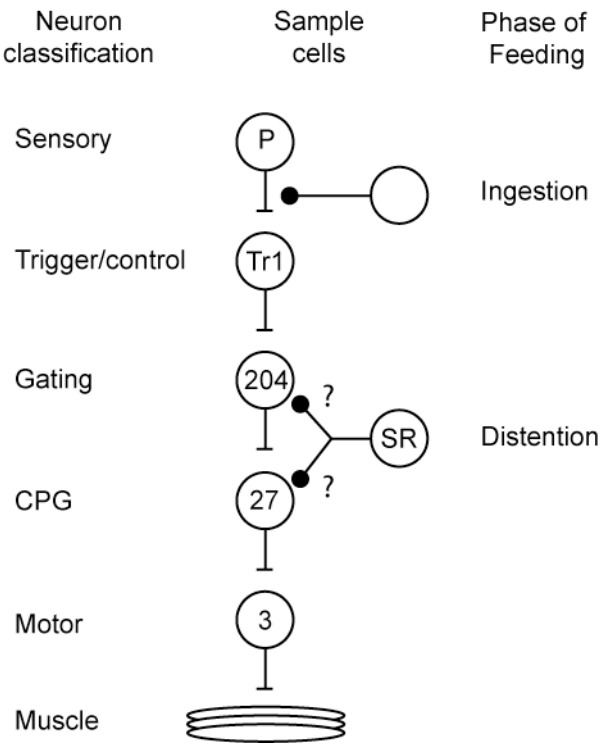


Fig. 3.6: Diagram summarizing the two ways that leech feeding inhibits the swimming circuit. Open circles represent cell populations. The letters and numbers inside the open circles indicate one identified neuron from that population type. Lines ending in bars represent excitatory connections and those ending in solid black circles represent inhibitory connections. The diagram shows the excitatory, feed-forward nature of the circuit but does not show the inhibitory interactions among the CPG neurons and between particular motor neurons. The inhibition from ingestion arises from an unknown source, probably chemical or thermal sensory pathways; it inhibits the P cell terminals (Chapter 2). The inhibition from distention originates from stretch receptors in the body wall and targets either the gating neurons or CPG neurons. Swim circuit connections taken from and reviewed in Kristan et al. (2005). P = Pressure mechanosensory P cell, Tr1 = Trigger neuron 1, 204 = Gating neuron 204, 27 = CPG neuron 27, 3 = Dorsal longitudinal muscle motor neuron 3, SR = stretch receptor, ? = potential connections. Kristan WB, Jr., Calabrese RL, Friesen WO (2005) Neuronal control of leech behavior. Prog Neurobiol 76:279-327.

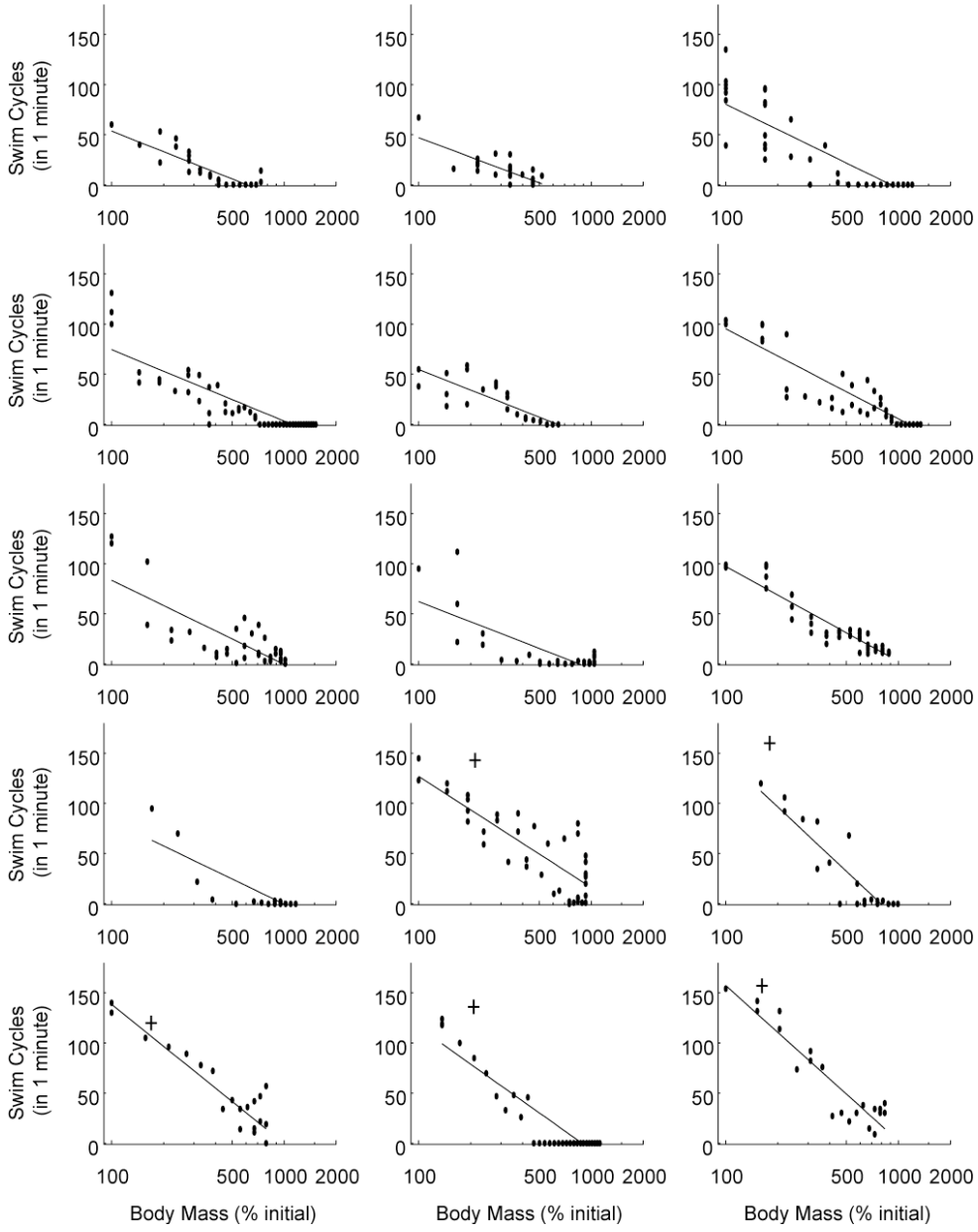


Fig. 3.7: Swimming cycles elicited following stimulation as a function of increases in body mass (plotted on a logarithmic scale) for individual animals with severed nerve cords (Figure 1A). Each plot shows all trials taken from one animal and the line is the linear regression for those data. These plots show that every animal decreased its swimming frequency with increased distention during feeding. The + symbols indicate data for the 5 leeches that were lanced following feeding.

4. Concluding Remarks

The chapters contained within this thesis show various strategies employed by the leech CNS to suppress competing behaviors during feeding. The importance and ability to initiate the appropriate behavior at the appropriate time is not unique to the medicinal leech. Therefore I propose that the strategies outlined in this thesis may be universal and applicable to a variety of species.

In Chapter 2, we demonstrated that the ingestion component of the leech feeding behavior causes an inhibition of the tactile sensory fibers of the P cells. This inhibition decreases the probability of release at P cells terminals and results in less excitation in downstream neurons responsible for the initiation of behaviors. Decreases in EPSP amplitudes where observed onto interneurons involved in the local bend response (LBI 212), shortening (S cell), swimming (Tr1 neuron), and a neuron of unknown function (AP neuron). Circumventing this sensory inhibition by direct stimulation of swim gating neurons restores swimming behavior in feeding leeches. The reduction of motor output upon nerve stimulation during feeding is mimicked through the bath application of serotonin. Serotonin also decreases EPSP amplitudes between the P cell and its postsynaptic partners. An increase in paired pulse ratios is observed during serotonin application, suggesting a presynaptic mechanism similar to what is observed during feeding.

Next we showed that distention inhibits the expression of swimming downstream of the sensory neurons (Chapter 3). By severing the nerve cords of otherwise intact leeches, we were able to initiate and study swimming in the posterior

region of the animal while the anterior portion of the leech fed. As the duration of a feeding bout increased, distention of the leech, measured as an increase in percent initial body mass, also increased. This increase in body mass is strongly correlated with a decrease in the number of swim cycles observed within a minute following stimulation. A semi-intact preparation was used to artificially distend the leech and give us greater control over the dynamics of distention. This preparation revealed that the inhibition arising from distention onto the swim circuit has a rapid onset (~1 second) and is readily reversible. Swimming episodes were prematurely terminated when distention was applied in the middle of a swimming bout, showing that distention can disrupt the maintenance of swimming after its initiation. Using isolated nerve cords and additional semi-intact preparations, we were also able to show that stretching of the central nerve cord and gut lining are not necessary for the distention to suppress swimming.

Together, the work described in Chapters 2 and 3 shows two distinct mechanisms that leeches use to suppress behaviors during feeding. Ingestion targets the sensory neurons of the leech and reduces the amplitude of graded reflexive behaviors like the local bend, and prevents the initiation of more complex rhythmic behaviors like swimming. As feeding progesses, distention targets the interneurons responsible for the maintenance of swimming behavior. Therefore, ingestion and distention work cooperatively to suppress swimming during feeding. However, ingestion and distention still remain separable anatomically since they rely on different groups of neurons. Therefore, the two inhibitory networks may be independently activated given appropriate environmental conditions. For example, it is important to

prevent all mechanically elicited behaviors that may interfere with the ingestion phase of feeding. Thus, an ideal strategy to prevent competing behaviors from being expressed is the direct inhibition of the mechanoreceptors. Upon the completion of feeding, leeches must recover their ability to respond to tactile stimulation, but still bias their decisions away from behaviors incompatible with a distended state; behaviors such as swimming. The feeding-induced distention can serve to suppress swimming without affecting other behaviors by specifically targeting the relevant interneurons. The distributed decision making network identified in the leech allows the animal to express individually or combine decision-making modules as the task or environment around them changes, thus ensuring the expression of appropriate behaviors at all times.