

Locomotion and its activation by dopamine in a simpler neural network

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Joshua Puhl

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Adviser: Dr. Karen A. Mesce, Ph.D.

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Summary of manuscripts and publications

-- Chapter Two is published in the Journal of Neuroscience and appears here with permission from the Society for Neuroscience:

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Chapter One:

Introduction

Overview of dissertation research

As a neuroscientist, I am interested in understanding the ‘language’ of the central nervous system (CNS) and how tiny changes in the voltage of populations of neurons, when coordinated with each other, lead to the expression of complex and dynamic behaviors. Studying how these phenomena regulate naturalistic behaviors at the level of individual cells and neural networks classifies me as a Neuroethologist.

Trying to understand the neural bases of behaviors can be daunting as it is often difficult to quantify behavioral outputs precisely. To help overcome this challenge, I have chosen to study locomotor behaviors. The reason for this choice is because: 1) locomotion is expressed universally among animals at some point in their lives, 2) compared to many other behaviors the outputs of locomotor behaviors are more easily quantifiable because of their stereotypy, 3) locomotion entails repeated/cyclical activity and thus can be studied routinely. By understanding the neural underpinnings of locomotion, I gain insights into a universally-expressed type of behavior and help reveal general strategies used by nervous systems to produce motor output; the nature of motor control disorders, such as Parkinson’s disease or spinal cord injury.

Architecture of motor control systems

The current consensus for the architecture of motor control systems, including those that generate locomotion, is that they often have a modular design (Grillner, 2003; Kristan et al., 2005). Figure 1-1 depicts a greatly simplified “systems-level” view of a motor control system. Although the different layers are not always as discretely separated as depicted, I will consider them to be separable unless mentioned otherwise.

At the ‘top’ of the hierarchy are elements that participate in decision-making processes and generate commands that activate or suppress certain behaviors.

Command elements are often located within higher-order regions of the CNS (i.e., the brain) (Dubuc et al., 2008; Jordan et al., 2008), but command elements have also been found located throughout the CNS (e.g., Weeks and Kristan, 1978; Pearson et al., 1985; Edwards et al., 1999). The command elements often perform other functions to modulate motor behaviors as well, but in the context of the architecture of motor systems, their primary function is to select and activate motor behaviors (Stein et al., 1999). Command elements often receive information from sensory and/or modulatory elements (Crisp and Mesce, 2004; Zelenin et al., 2007).

Sensory elements gather information about the outside world. In the context of motor systems, animals use this information to select and modulate all of the other components of the control scheme (reviewed: Stein et al., 1999). Understanding sensory systems is a large and active area of neuroscience, however, it is not the focus of my thesis. Regardless, sensory inputs are an important component of motor-control systems (Pearson, 2000). Motor systems are aware of and integrate information from a variety of sensory information streams including proprioceptive, mechanosensory, visual, chemosensory and auditory stimuli (Wallen, 1994; Stein et al., 1999). The specific types of information and their relative importance to a given animal's motor-control infrastructure varies among different species and is tailored to each animal's specific environment, anatomy, behaviors, etc.

Modulatory elements can affect motor systems in a variety of ways (Selverston, 1985). For example, a neuromodulator can promote or inhibit motor outputs (e.g., Cazalets et al., 1990; Crisp and Mesce, 2004; Chapter 2) or alter the timing elements of ongoing rhythmic activity (e.g., Svensson et al., 2003). Neuromodulators often are released to act in a paracrine fashion (e.g., Sombati and Hoyle, 1984) or can be released into the blood stream to act in a hormonal capacity (e.g., Willard, 1981;

reviewed: Gilbert, 2009). The temporal characteristics of modulators vary from very short (< 1 sec.) to very long (days/weeks). Modulatory elements also can influence the function of all of the other components in the motor control infrastructure (Kristan et al., 2005; Stein et al., 1999).

The three elements of the motor control infrastructure mentioned so far primarily function to select, initiate and shape motor behaviors. The last two layers are responsible for generating the actual movements. The motoneurons (MNs) are a specific type of neuron that communicates directly with muscle fibers (Kandel et al., 2000). The MNs are directly responsible for generating muscle contractions. Motoneurons can be influenced by command, modulatory, sensory and pattern-generating elements of the motor control infrastructure (Stein et al., 1999).

The last layer that I will discuss is the central-pattern-generating layer. This layer establishes the basic rhythmicity that forms the core of cyclic motor behaviors, including locomotion (Marder and Calabrese, 1996; Marder and Bucher, 2001; Grillner, 2003). The rhythm-generating layer can be activated or modulated by the command, sensory or modulatory elements. This layer is made up of specialized neural circuits that are capable of generating rhythmic neural activity in the absence of patterned inputs and they are called central pattern generators (CPGs) (Marder and Calabrese, 1996). The organization, localization and underlying mechanisms of CPGs is an active area of research and is the emphasis of Chapter 2 of my thesis as well as an important component of Chapter 3. I will discuss the inner-workings of CPGs, their location within the CNS and more in subsequent sections of this chapter.

Model systems for studying locomotion

To satisfy my own intellectual curiosities it would be ideal for me to understand the underlying neural mechanisms of locomotion in humans. I am fascinated by the complexity of the control systems that allow us to walk. Furthermore, gaining knowledge of the neural mechanisms that generate our movements paves the way for treatments for diseases of motor control such as Parkinson's and spinal cord injuries. Severe ethical and technical limitations, however, preclude invasive human studies. Instead, simpler more experimentally accessible animal models are used, which possess certain features common to more complex animals. For example, to be relevant, the locomotion in question must be generated by CPGs (Guertin, 2009). Furthermore, if considering an invertebrate model organism, it should possess a metameric arrangement of its body to mirror a vertebrate plan of organization. In the following text several model systems will be introduced that have provided valuable insights into the neural underpinnings of locomotion.

The cat is a classical and important model in which locomotion has been studied. During the early 1900's, studies in the cat were performed to justify the existence of centrally-generated rhythmic movements of the legs during walking (Brown, 1911). Prior to this important discovery, walking movements were thought to be generated by a complex set of chained reflexes (Sherrington, 1906). Originally, Sherrington proposed that walking movements were generated in the spinal cord although he concluded that they were generated by proprioceptive stretch receptors interacting with motoneurons and spinal reflex circuits. He came to this conclusion upon placing a cat whose brain was severed from its CNS (i.e., spinalized) on a treadmill, where he observed alternating leg movements that resembled walking. In 1911, Brown showed that these walking-like movements were still expressed after severing the afferent fibers in the legs of

spinalized cats. Brown proposed that neural circuits within the spinal cord generated the basic signals for walking movements and the concept of a CPG was conceived. Since that time, cats have been an instructive model for understanding the kinematics of walking movements, the role of sensory information in modulating locomotion and the contributions of higher-order brain regions on motor control (reviewed in: Stein et al., 1999)

More recently, rodents have become a favorable model for studying locomotion. Rodents are also walking mammals that roughly have a similar functional neuroanatomy to cats and humans. More importantly, the mouse genome has been sequenced and many techniques have been developed to manipulate the genome of this animal (Eisen, 2005). For example, genetic 'reporter' lines have been created that express fluorescent markers, like Green Fluorescence Protein (GFP), in a specific subset of cells within the CNS of the mouse (reviewed: Kiehn and Butt, 2003). These reporter lines facilitate targeting of specific cell types for neural recordings. Using contemporary genetic tools, researchers also can insert conditionally active ion channels, such as light-activated channel rhodopsin genes, into specific population of neurons, which permits the at-will activation and deactivation of specific cell types (Hägglund et al. 2010). Although this technology is relatively new, it has provided key insights into understanding mammalian nervous system function.

In addition to a rich set of genetic tools, the CNS tissues of rodents, in particular neonates, can be removed from the body while remaining viable and able to produce locomotor-like rhythmic activity over a period of several hours (e.g., Zhong et al., 2007). This removal permits greater and more simplified access to the various parts of the CNS as well as reduces instabilities caused by movements of the animal without complete degradation of neural activity. These techniques and powerful genetic tools have

allowed scientists to begin investigating the activities of individual cells or classes of cells (examples: Dougherty and Kiehn, 2010; Zhong et al., 2010) during locomotor-like activity as well as understanding better their physiological properties (Zhong et al., 2006; Diaz-Rios et al., 2007). Increased access of isolated neural tissues has also facilitated the use of calcium-sensitive dyes to monitor the activities of many cells simultaneously (Bonnot et al., 2009; Zhong et al., 2010). Although these technologies are shedding light on the inner-workings of neural networks that control locomotion, to date, studies in the rodent have focused only on the contributions of large nuclei and/or classes of neurons. Contributions of a sub-class of neurons or individual neurons have been limited, primarily by the technical limitations of the methods.

A particularly instructive model system for studying locomotion and motor control at the systems and cell-class level has been the lamprey, an evolutionarily basal vertebrate (Grillner and Wallen, 2002; Grillner, 2003). Lampreys are primitive vertebrates that possess a spinal cord and many higher-order brain structures similar to those found in mammals, however, they possess relatively fewer neurons within their CNS than mammals. Similar to rodents, the lamprey spinal cord can be removed from the body, and locomotor behavior (i.e., swimming) can be studied *in vitro* (McClellan and Grillner, 1984). The lamprey is a good balance between complexity and accessibility in a vertebrate animal. Studying individually identifiable neurons and how they integrate into neural circuits is still limited. Regardless, a number of insights into understanding the organization and function of the CNS has been elucidated in lampreys (Stein et al., 1999; Grillner, 2003). Many of the first studies of CPG organization in vertebrates were characterized in lampreys (Grillner et al., 1988). Compared to other vertebrates, more is known about the underlying circuitry and cells involved in generating locomotor movements in lamprey (reviews: Buchanan, 2001; Grillner, 2003; Grillner, 2006; Dubuc

et al., 2008). Tools like computational modeling have been particularly helpful to bridge the gaps in knowledge of lamprey CPGs and locomotor control systems. Ambitious computational models have been created that have helped to validate and integrate systems-, network- and cellular-level studies (Kozlov et al., 2009). The lamprey CNS has also been used to study sensory-motor integration (Wallén, 1994; Dubuc et al., 2008) and descending control of locomotion (Dubuc et al., 2008).

Studies of vertebrates, as described above, have been instructive in our understanding of the organization of the CNS at a systems and network level of detail. In order to study the neural underpinnings of locomotion at a finer level of detail, such as how individual neurons communicate to generate behaviors, simpler invertebrate model systems have been used (Selverston, 1985). Invertebrates possess fewer neurons within their CNS than vertebrates and it is more feasible, in general, to relate neural activities of classes of neurons or in some cases individual neurons to behaviors. A number of non-insect invertebrates, generally, lack a formidable blood-brain-barrier making delivery of neuroactive substances easier. In many invertebrate preparations, neurons are identifiable between individuals. Similar studies at this level of detail are often too difficult or impossible in vertebrate preparations (Grillner, 2003). The important and salient contributions of invertebrate studies to our current understanding of CPGs, their activation and intersegmental coordination will be reviewed in the text below.

The European medicinal leech: An almost ideal model for studying locomotion at the cellular and network levels of detail.

One particularly useful and well-established model for studying the cellular and network bases of locomotion is the European medicinal leech (*Hirudo verbena*; Siddall et al., 2007). Medicinal leeches are metamerically segmented sanguivorous (i.e., blood-

eating) annelid worms. The medicinal leech has been the focus of my thesis research for a number of reasons, which are outlined below. Much of the information in this section comes from a recent and comprehensive review by Kristan et al. (2005) and/or textbook on leeches by Muller et al. (1981).

Several characteristics of the leech CNS make it an attractive model for studying the neural bases of locomotion. The CNS comprises an anterior compound cephalic ganglion (i.e., brain), 21 segmental ganglia (one per segment) and a compound terminal ganglion. These structures are joined together forming the ventral nerve cord by bundles of neuronal fibers called connectives. The connectives consist of two, large, laterally-situated (hemi) connectives and a thin singular median 'Faiver's nerve'. The entire CNS contains an estimated 10,000 neurons which is orders of magnitude less than even the simplest vertebrates. In general, the neuronal somata are situated within ventral and dorsal layers while the neuronal processes form a complex neuropil between the somatic layers. The dorsal and ventral layers of somata are divided into "packets" by the membranes of giant glial cells. The vast majority of synapses of the CNS occur within the neuropil.

The cephalic ganglion comprises a compound supra-esophageal ganglion and the sub-esophageal ganglion (SEG). The SEG is partitioned into four neuromeres, named R1-R4, by the membranes of giant glial cells. The dorsal surface of the second, third and fourth neuromeres are subdivided further into two sub-neuromeres. The subneuromeres are identified with 'a' and 'b' suffixes (e.g., R3b).

Each midbody segment contains a single segmental ganglion. A pair of (dorsal and ventral) nerve roots, containing mixed motor and sensory axons, protrudes from the left and right sides of each ganglion. These nerve roots, *via* a number of stereotypical branches, innervate the tissues, musculature and cuticle (i.e., skin) of a given segment.

The segmental ganglia are named M1-M21, corresponding to the segment in which they are located. Each segmental ganglion contains about 200 pairs of cells that are typically segmentally iterated.

The relative location, arrangement, position and size of neuronal somata in both the cephalic ganglion and the segmental ganglia are remarkably similar between and across individuals. Furthermore, the morphological and physiological signature of individual 'identified' neurons is the same between and across individual leeches. For example, the paired ventral lateral circular excitor (CV) motoneuron (MN), which activates a subset of circular muscle fibers along the lateral portion of the ventral body wall is found in a similar location in all segmental ganglia within a given leech. Each segmental ganglion within a population of leeches possesses a pair of CV MNs that are located in the same general vicinity within the ganglion and possesses a similar unique physiological signature and morphology (Stuart, 1970).

Accessing the various components of the leech's CNS is relatively easy compared to many other animal systems. The entire CNS can routinely be removed from the body and remains viable for up to 6 or more hours *in vitro* if maintained in fresh oxygenated physiological saline. When removed, the activities of individual neurons and nerve bundles can be monitored using electrophysiological recording methods. Although the body is no longer available for observing overt locomotor behaviors, the neural correlates of locomotion or 'fictive locomotion' can be monitored by recording from the peripheral nerve roots of segmental ganglia. In particular, the activity of the dorsal longitudinal excitor (DE-3) MN is routinely monitored as it is rhythmically active during both primary forms of locomotion (see below). DE-3 was selected as the *de facto* locomotor monitor signal; its axon resides within the dorsal posterior (DP) nerve root of each ganglion and the action potential generated by DE-3 is the largest observable unit

in extracellular recordings of the DP nerve (Kristan et al., 1974). It has been shown repeatedly to serve as a monitor cell for locomotion (Kristan et al., 2005).

The leech expresses two primary forms of locomotion, swimming and crawling. Swimming is an aquatic form of locomotion characterized by repeated sinusoidal undulations of the elongated and flattened body (Kristan et al., 2005). Swimming is the faster of the two forms of locomotion with cycle periods ranging from ca. 0.5-2.0 Hz. As is the case with almost every locomotor behavior studied to date, the core rhythmicity for swimming is generated by a CPG (Kristan and Calabrese, 1976). Each segmental ganglion is capable of generating swimming motor outputs, however, the core rhythm-generating circuitry is heterogeneously distributed (Hocker et al., 2000). The constituents and underlying circuitry of the rhythm-generating elements for swimming have been characterized (Brodfuehrer et al., 1995a; Brodfuehrer and Thorogood, 2001; Kristan et al., 2005). Additionally, other cells have been identified that control the swim CPGs. Located within each ganglion from M9-M16, cell 204 or the swim-gating neuron is a command-like cell whose activity is necessary and sufficient for the production of fictive swimming (Weeks and Kristan, 1978). Furthermore, the serotonergic cells 21 and 61 can activate swimming (Nusbaum and Kristan, 1986) and serotonin when applied exogenously decreases the threshold for swim activation (Willard, 1981). Several descending higher-order neurons located in the SEG have been identified that initiate (Tr-1, Tr-2, R3b-1, SE1; Brodfuehrer and Friesen, 1986a; Brodfuehrer et al., 1995b; Esch et al., 2002) or terminate (Tr-2, SIN1; Brodfuehrer and Burns, 1995; O'Gara and Friesen, 1995) swimming. Recently, an ascending projection neuron located in M21 (called E21) was identified that rapidly activates fictive swimming (Mullins et al., 2010). These higher-order cells likely act as a population to control swimming behaviors

(Kristan et al., 2005; Chapter 4). Their specific contributions to the control of swimming are described in more detail in later sections of this chapter.

The other primary form of locomotion expressed by the leech is crawling and is the focus of my thesis. Crawling is characterized as the alternating elongation and contraction (i.e., shortening) of the body with coordinated sucker attachments that drag the leech's body across a substrate (Stern-Thomlinson et al., 1986). Importantly, the movements of the body during crawling propagate from anterior to posterior in a metachronal wave. Crawling, although most often expressed in terrestrial environments can also be expressed underwater (Puhl and Mesce, personal observation). The kinematics of crawling have been studied and compared to swimming is a more flexible behavior (Stern-Tomlinson et al., 1986; Cacciatore et al., 2000). For example, a leech can terminate crawling mid-elongation and begin a series of whole-body bending movements without having to reconfigure its body posture. Furthermore, after completing the whole-body bending "sub-behavior," the animal can continue elongation and complete the paused crawl cycle. Eisenhart et al. (2000) determined that crawling is generated by a CPG. The activity patterns of some segmental interneurons and MNs during crawling have been reported (Baader, 1995; Eisenhart et al., 2000), however, no members of the crawl oscillator have been identified.

Central pattern generators: the core of locomotion

As was introduced above, specialized neural circuits that are capable of generating patterned output in the absence of patterned (sensory or other) inputs are called CPGs. These circuits form the rhythmogenic kernel of repetitive behaviors, such as locomotion. A number of neural mechanisms have been proposed for the rhythm-generating capacity of CPGs (reviewed: Marder and Calabrese, 1996; Marder and

Bucher, 2001). Two cellular mechanisms have been studied in greatest detail; they depend on the intrinsic properties of the neurons within the CPG and/or their synaptic connectivity. In a 'pacemaker' network, one or more neurons possess intrinsic properties that cause them to produce bursts of action potentials when they are activated. The rhythmic activity of these pacemaker neurons drive the rest of the cells in the CPG, which are not intrinsically rhythmic, but play a role in shaping the rhythmic outputs of the CPG. Examples of pacemaker-driven CPGs include the pyloric CPG of the crustacean stomatogastric nervous system (Marder and Eisen, 1984) and the respiratory CPG of many vertebrates (Smith et al., 1991). The other major mechanism to generate rhythmicity within a CPG is called the 'network oscillator.' Rhythmic activity in this case arises as a property of the synaptic interactions among neurons within the CPG (Calabrese, 1998). In a network oscillator, none of the CPG members are intrinsic oscillators. The simplest example of a network oscillator is the half-center one. A half-center oscillator is formed by two neurons that mutually inhibit each other *via* symmetric inhibitory synaptic connections. When one neuron is activated, it inhibits the other. At some point, based on the intrinsic properties of the cells, the second neuron 'escapes' from inhibition and becomes excited, thus inhibiting the first neuron (reviewed: Calabrese, 1998; Marder and Bucher, 2001). CPGs that underlie many locomotor behaviors reflect these types of network oscillators. For example, the CPG that controls swimming behavior in the medicinal leech is a type of network oscillator (Brodfuehrer et al., 1995a; Kristan et al., 2005) as is the CPG that generates swimming in the lamprey (Grillner, 2003).

The half-center or network oscillator theories for CPG rhythm generation have provided a solid foundation for studying simpler locomotor behaviors. Most locomotor behaviors, especially those in vertebrate animals with complex locomotor organs (i.e.,

limbs), require a complex pattern of activation of multiple muscle groups that transcends the simple alternation of a flexor-extensor pair (Stein et al., 1999). To address this complexity, two main CPG architectures have been proposed. The first is called the unit-burst-generator (UBG) theory and was proposed by Grillner (1981). Under this theory, subsets of muscles and MNs for a given complex locomotor behavior are controlled by a single UBG. A given UBG often will control a single flexor-extensor pair or some other small grouping of antagonist muscles. The individual UBGs that control the muscles for a given behavior are coupled to one another and their activities are coordinated by means of their synaptic connectivities. Together, the activities of multiple UBGs produce the intricate phase relationships seen in muscle activation of complex locomotor behaviors. The other major CPG architecture proposed by McCrea and Rybak (2008) is known as the 2-layer CPG theory. In this theory, the CPG is segregated into rhythm-generating and pattern-generating layers. The rhythm-generating layer produces the core timing elements of the behavior. The pattern-generating layer is responsible for directly controlling the MNs and muscles. This theory was originally proposed to account for observations in which changes in the activities of individual pools of MNs were observed without alteration of the rhythm itself. This model allows for factors external to the CPG, such as sensory information, to modulate the properties of the individual phases of a given locomotor step-cycle without resetting the overall rhythm. Data from locomotor behaviors in humans corroborate a potential separation of rhythm- and pattern-generating functions (reviewed by Ivanenko et al., 2006).

In addition to forming the core of locomotor behaviors, CPGs drive other rhythmic behaviors, like respiration/heartbeat (von Euler, 1983; Calabrese, 1998; Gdovin et al., 1998), escape (Katz, 1998), feeding (Wentzell et al., 2009), communication (Hoy, 1978; Barlow and Estep, 2006) and mating (Carro-Juarez et al., 2003). Furthermore, certain

cognitive processes appear to rely on oscillatory (i.e., rhythmic) activity within the brain (reviewed: Grillner et al., 2005; Wang, 2010). These oscillations could perhaps be generated by CPGs or themselves be CPGs, as some of the mechanisms elucidated to generate these oscillations are similar to those described above (Wang, 2010). The fact that CPGs are part of the foundation of many behaviors makes studying them important in the context of locomotion and in understanding the neural mechanisms of behaviors, in general.

Anatomical location of central pattern generators

A critical issue in studying CPGs is establishing the location and extent of the rhythm-generating neural circuitry for a given behavior. Some key questions that have been investigated include: How are CPGs distributed in metamericly segmented animals, like mammals, fish and leeches? Is the rhythm-generating kernel distributed across multiple segments or does each segment contain CPGs for locomotion? Studies of walking vertebrates, primarily cats and neonatal rodents, suggest that the cervical and lumbar enlargements of the spinal cord contain the core CPG circuitry for walking movements of the fore and hind limbs, respectively (Kiehn, 2006). In the literature, however, more attention has been paid to lumbar locomotor networks. A consensus has emerged that the rhythm-generating circuitry for controlling the hind limbs is localized within, and distributed throughout, the lumbar spinal segments, although several theories concerning the rostrocaudal distribution of CPGs within the lumbar enlargement have been proposed (Kjaerulff and Kiehn, 1996; Bertrand and Cazalets, 2002; Berkowitz, 2004; Christie and Whelan, 2005). In the lamprey, short segments of the spinal cord as small as 1.5-2 segments can produce fictive locomotor activity (Grillner et al., 1991), suggesting that a single segment or a small number of segments may contain a complete CPG for locomotion. This difficulty in determining the exact extent of

locomotor CPGs arises because of the anatomical compactness of spinal cord tissues and the inherent complexities of the locomotor behaviors they control.

Studies in invertebrate animals have been more enlightening as to the extent and localization of locomotor CPGs due to the relative simplicity of their locomotor behaviors and the underlying neuroanatomy. In the locust, the cells that make up the flight CPG are distributed among several segments (Robertson and Pearson, 1983; Robertson and Pearson, 1985). Conversely, in the crayfish, a complete CPG for movement of one swimmeret is housed within a single abdominal (A2-A5) hemi-ganglion (Murchison et al., 1993). In stick insects, an isolated body segment, with only a single leg attached, can produce walking movements, suggesting that a CPG for walking is housed in a single segment (Borgmann et al., 2009). In the leech, individual segmental ganglia house a CPG for swimming, however, they are heterogeneously distributed throughout the anterior two-thirds of the CNS (Hocker et al., 2000). I determined that a complete CPG for crawling is housed within a single ganglion and that each and every segmental ganglion within the leech CNS contains a complete CPG for crawling (Chapter 2).

The distribution of multiple CPGs to control locomotion creates the fascinating problems of activating and coordinating their activities for the productive movements of complex locomotor organs. The following sections will review these ideas.

Activation of CPGs

Activation of CPGs is critically important for the normal expression of locomotor behaviors. CPG activation occurs by means of several mechanisms including, interneuronal activation by gating cells and/or command-like cells, activation by sensory systems and activation by neuromodulators like biogenic amines (reviewed: Selverston, 1985; Stein et al., 1999; Pearson, 2000).

Activation of CPGs by local or command-like interneurons appears to be a key mechanism in the initiation of locomotion in many animals (Heinrich, 2002; Dubuc et al., 2008; Jordan et al., 2008). In vertebrates, populations of command-like interneurons located in the brainstem and midbrain activate locomotor activity (Whelan, 1996). Stimulation of the midbrain, specifically the mesencephalic locomotor region (MLR) of decerebrate cats reliably activates treadmill walking (Shik et al., 1966). Similar findings have been reported in neonatal rodents (Hägglund et al., 2010). Studies by McClellan and Grillner (1984) determined that stimulation of a midbrain area deemed to be homologous to the MLR in lampreys elicited fictive locomotion. Unfortunately, because the exact location and constituents of locomotor CPGs in these higher animals have not been elucidated, the specific mechanisms of how higher-order centers activate these circuits are not known. What is known is that neurons within the MLR project onto reticulospinal neurons in the brainstem locomotor regions, which then project to the spinal cord (reviewed: Stein et al., 1999).

A number of studies have investigated the contributions of interneurons for activating and shaping locomotion in invertebrate organisms (e.g., Brodfuehrer and Thorogood, 2001; Heinrich, 2002). These studies have provided, in many cases, more detailed information about how interneurons drive and interact with CPGs. A classic example is that of several projecting interneurons reported by Wiersma and Ikeda (1964) that, when stimulated, produced rhythmic movements of the swimmerets in crayfish. A group of interneurons located in the mesothoracic ganglion of locusts, named the 404 interneurons, activate flight behavior when depolarized (Pearson et al., 1985). These neurons were also found to be tonically active during ongoing flight and were deemed to be necessary and sufficient for flight behavior. A pair of identified projecting interneurons located in the brain of crickets can activate walking behavior and were

found to be active just prior to and during walking movements (Böhm and Schildberger, 1992).

A number of identified interneurons in the leech have been studied in detail that activate and drive locomotor behaviors (reviewed by Kristan et al., 2005). In the cephalic ganglion, as mentioned previously, the descending projecting neurons Tr-2 and SE1 can trigger fictive swimming behavior upon brief stimulation (Brodfuehrer and Friesen, 1986a; Brodfuehrer et al., 1995b) and Tr-1 can activate either fictive crawling or swimming when stimulated (Brodfuehrer et al., 2008). Another descending projection neuron, R3b-1, activates (Esch et al., 2002) and sometimes gates (Chapter 4) fictive and overt locomotion (crawling or swimming depending on the context) when stimulated. In addition to projection neurons located in higher-order CNS structures, the segmental interneurons 204, 21 and 61 all activate fictive swimming upon their depolarization (Weeks and Kristan, 1978; Nusbaum and Kristan, 1986) as does the ascending projection neuron located in the most caudal segmental ganglion, E21 (Mullins et al., 2010). Some of the synaptic targets of 204, 21 and 61 are members of the swim CPG.

A key fact often overlooked in the context of central pattern generation is that sensory inputs, although not *necessary*, play a critical role in driving and maintaining locomotion (Stein et al., 1999; Pearson, 2000); this maintenance can be looked upon as ongoing activation. Proprioceptive sensory information is particularly important and has been well studied (Stein et al., 1999). In the cat, walking can be initiated in spinalized animals *via* the activation of proprioceptive receptors (i.e., stretch receptors), a consequence of placing the animal on a moving treadmill (Sherrington, 1906; Whelan, 1996; Stein et al., 1999). Specifically, one method for initiating a step in these animals is to induce an extension of the hip, which is necessary for initiating the flexion phase of the walking step-cycle (Grillner and Rossignol, 1978). Once this stepping occurs,

walking behaviors ensue when the animal is placed on a treadmill. Interestingly, data obtained in the stick insect qualitatively reflect those reported in the cat (Bässler, 1993). Unloading of a leg, typically by extension of the most proximal joint, induces swing-phase (i.e., flexion) leg movements. These movements are regulated by two groups of proprioceptors: the load-sensitive campaniform sensilla, which inhibit the initiation of flexion of the proximal joint and the femoral chordotonal organ, which facilitates flexion of the proximal joint in the absence of load on the leg.

The roles of proprioceptive and environmental sensory stimuli have been studied in detail in the locust. Proprioceptive information from wing tegulae, and stretch receptors, which are excited by wing depression and elevation respectively, help to maintain the rapid fluttering of the wings. The tegulae do this by causing a rapid depolarization of elevator MNs when they are activated by depression of the wing (Wolf and Pearson, 1988). Conversely, wing stretch receptors that sense wing elevation help to prevent the inactivation of depressor MNs (Pearson and Ramirez, 1990). When the connections from these structures are severed from the CNS, flight movements continue, however, the frequency of wing beating decreases and eventually flight stops, even in the presence of a wind stimulus. Furthermore, in the locust, flight is activated and maintained by stimulation of the wind-sensing facial setae (Weis-Fogh, 1949). Some of these setae were shown to make direct connections with flight MNs (Bacon and Tyrer, 1978; Simmons, 1980). It was shown later that wind stimulation could excite the aforementioned 404 interneurons, which excite MNs that elevate the wings (Pearson et al., 1985). Elevation of the wings *via* either of these mechanisms excites wing stretch receptors and facilitates the initiation of alternating elevation and depression of the wings (i.e., flight).

Studies of the medicinal leech have led to a cellular-level understanding of sensory activation of the CPG for swimming. Intracellular stimulation of the segmental touch- and pressure-sensitive neurons as well as nociceptive neurons (i.e., mechanoreceptors) activates swimming behaviors (Debski and Friesen, 1986). This effect is not, however, a direct one. Pressure-sensitive and nociceptive mechanosensory neurons excite the descending swim trigger neuron Tr-1, which excites the swim gating neurons 21, 61 and 204 (Brodfuehrer and Friesen, 1986b; Gilchrist and Mesce, 1997). These gating neurons then excite members of the swim CPG (Weeks, 1982; Nusbaum et al., 1987). Although body-wall stretch receptors alone are not sufficient to activate the swim CPG (Debski and Friesen, 1986), a combination of stretch receptors and mechanoreceptors activate the swim CPG in animals whose inter-ganglionic connectives are severed (Yu and Friesen, 2004). This sensory information is effective enough not only to activate swimming movements in ganglia posterior to the injury by driving the CPG, but also to maintain appropriate intersegmental coordination.

Activation of CPGs by neuromodulators, is the last avenue that I will explore. Although not specifically a locomotor CPG, studies of the crustacean digestive CPG (i.e., the stomatogastric nervous system) have been instrumental to our understanding of the roles of neuromodulators on pattern generating networks (reviewed: Selverston, 1985). The stomatogastric ganglion (STG) when detached from other CNS structures and sensory information is often quiescent, however, introducing neuromodulators is sufficient to induce rhythmic activity (Beltz et al., 1984; Selverston, 1985). For example, application of pilocarpine, an acetylcholine receptor agonist, induces rhythmic motor outputs in quiescent preparations (Marder and Paupardin-Tritsch, 1978). Application of the biogenic amine DA also induces robust rhythmic pyloric activity (Marder and Eisen, 1984).

CPGs that underlie locomotion are also susceptible to activation by neuromodulators. Introduction of pilocarpine to isolated ganglia of stick insects, for example, can activate locomotor-like patterns of neural activity (Büschges et al., 1995). Application of the peptide neurotransmitter proctolin to isolated crayfish abdominal ganglia reliably induces fictive swimmeret beating (Acevedo et al., 1994).

The biogenic amines, in particular serotonin (5HT), octopamine (OA), and dopamine (DA) have been studied in the context of CPG activation (see examples below). Two classical studies, conducted in invertebrates, have established that these substances play a role in the activation of CPGs. The first showed that octopamine, when ionophoretically injected into the metathoracic ganglion of locusts, induces either flight or stepping movements, depending on the location of injection (Sombati and Hoyle, 1984). In the same study, the authors proposed a hypothesis for octopamine being a natural route for activation of motor behaviors. A similar study of the medicinal leech established that 5HT application to an isolated but intact CNS induced fictive swimming in the absence of other stimuli (i.e., electrical stimulation of nerves) (Willard, 1981). Willard also proposed that activation of swimming by 5HT had behavioral relevance. He reported that stimulation of 5HT-containing neurons had similar effects to exogenous application and also showed a correlation between the occurrence of swimming behavior and 5HT levels in the blood. O’Gara et al. (1991) provided indirect evidence of CPG activation by 5HT by depleting amines *via* treatment of whole leeches with reserpine. Reserpine treatment reduced or eliminated locomotor behaviors and bath-treatment of 5HT restored swimming behaviors.

In vertebrate animals, biogenic amines can also influence the activation of CPGs although mixtures of multiple amines and/or excitatory amino acids are often necessary. In the neonatal rat spinal cord, application of a mixture of 5HT and *n*-methyl-D-aspartate

(NMDA; an excitatory amino acid) induces slow locomotor-like rhythmic activity. When norepinephrine (NE) was added to the 5HT/NMDA mixture, faster locomotor-like rhythmic activity was observed (Cazalets et al., 1990; Cazalets et al., 1992). Because their experiments were done in isolated spinal cord tissues in which sensory inputs were absent, the authors concluded that these mixtures of neuroactive substances were acting directly on the locomotor CPGs. Similar effects of 5HT/NMDA mixtures were found in lamprey (Harris-Warrick and Cohen, 1985).

The last biogenic amine that has received considerable attention in the context of locomotion is DA. In general, less is known about the role DA plays in activation of locomotor CPGs, however, it is clear that DA is a universal modulator of locomotor behaviors (Kandel et al., 2000). In humans, DA has been implicated in the control of locomotion. The best evidence of this comes from diseases of motor control where DA-synthesizing neurons are impaired or destroyed such as in Parkinson's disease (Davie, 2008). Although few mechanisms are understood about how DA affects human CPGs, it is clear that when DA levels are decreased severe deficits in locomotion and in the ability to generate voluntary movements develop. In the neonatal rodent spinal cord, DA acts in concert with 5HT and NMDA to activate fictive locomotor-like bursting (i.e., CPG outputs) (Whelan et al., 2000). Application of DA alone activates slow, irregular bursting activity in rat isolated spinal cords (Kiehn and Kjaerulff, 1996). In *in vivo* rodent spinal cord preparations, where sensory information is present, administration of D1-like DA receptor agonists induce rhythmic locomotor-like movements, although it is not clear whether these pharmacological agents are acting directly on the locomotor CPGs (Lapointe et al., 2009). In the isolated lamprey spinal cord, DA modulates ongoing fictive swimming, but alone it is not sufficient to induce CPG activation (Svensson et al., 2003).

Surprisingly, the precise behavioral role for DA in controlling locomotion has not been elucidated for the vast majority of invertebrate animals studied. In *Drosophila*, locomotion is reduced and often abolished when the gene for tyrosine hydroxylase, the rate-limiting DA synthetic enzyme, is knocked-out by mutation (Pendleton et al., 2002). DA has also been shown to be important for locomotion in the primitive planarian flat worm (Nishimura et al., 2007).

Recent work in the medicinal leech has provided exciting new insights into the effects of DA on CPGs for locomotion. The swim-terminating “trigger” neuron, Tr-2, is dye coupled (i.e., electrically coupled; Fan et al., 2005) to the lateral DA-synthesizing neurons in the cephalic ganglion (Crisp et al., 2002; Crisp and Mesce, 2004). This result establishes an anatomical association between a descending command-like system and a modulatory system. This is one of only a few examples in any animal system that provides a direct connection between neuromodulatory neurons and higher-order ‘command-like’ systems. Furthermore, Crisp and Mesce (2004) also showed that exogenous DA application to isolated CNS preparations terminates ongoing fictive swimming and suppresses future episodes. My work has elucidated that exogenous DA application activates the CPG for crawling in the intact but isolated CNS and does so in as little as a single isolated segmental ganglion (Chapter 2). These studies provide clear evidence that DA can both inhibit and activate locomotor CPGs. These studies also establish a potential behavioral role for DA as a decision-making cue for the selection of locomotor behaviors, an idea that I will discuss further in later sections of this chapter.

Intersegmental coordination of locomotor CPGs

The locomotor organs (i.e., limbs, body segments, etc.) of most animals are complex and comprise multiple segments or joints. Often times, multiple CPGs or ‘unit

oscillators' are employed in order to effectively control these complex structures and behaviors (*leech*: Chapter 2; *walking stick insect*: Büschges, 2005; *lamprey*: Grillner 2003; *walking vertebrates*: Grillner, 1981). This fact presents a significant challenge for the CNS because the various unit oscillators must be activated in a particular order with proper timing so that productive movements are generated. Thus, the activities of the CPGs must be coordinated (Grillner, 2003). In metameric animals, two basic strategies have been proposed for inter-oscillator coordination: more tightly and more flexible coupling. Often times, as coupling becomes more flexible, it is at the expense of stereotypy and relative simplicity.

In more tightly coupled systems, oscillator coordination is an emergent property of the locomotor CPGs themselves, or the coupling mechanism is inseparably associated with the CPGs (examples: Cohen and Wallén, 1980; Cang and Friesen, 2002; Smarandache et al., 2009). In tightly coupled systems, when a locomotor behavior is activated, the entire system (i.e., the whole body) participates in the behavior. Thus, it is often difficult or impossible to activate only a single CPG (i.e., uncouple them). A well-studied model of a more tightly coupled locomotor system is swimmeret beating in crayfish. Each abdominal ganglion contains a left and right CPG that controls the pair of swimmerets associated with that segment (Murchison et al., 1993). The beating of the swimmerets progresses from posterior to anterior with predictable intersegmental phase delays (Ikeda and Wiersma, 1964). The intersegmental coordination of the swimmerets is mediated by a small population of intersegmental interneurons that establish an excitability gradient (Mulloney, 1997; Namba and Mulloney, 1999). Intersegmental coordination self-organizes upon simultaneous activation of the all of the abdominal ganglia (Mulloney, 1997) or when only a single ganglion is activated (Acevedo et al., 1994). Furthermore, intersegmental

coordination is maintained when a single ganglion is deactivated by blocking local synaptic activity in that ganglion (Tschuluun et al., 2001). The ability of the ganglia to self-organize their activity between segments along with the apparent inability to uncouple them by interrupting a segmental pair of CPGs suggests that coupling is locally organized and tightly associated with the segmental oscillators.

The CPGs that control swimming in the medicinal leech are also more tightly coupled. When a single CPG is stimulated to swim in an isolated CNS preparation, either by shocking a peripheral (i.e., DP) nerve or by activating an identified mechanosensory neuron (Debski and Friesen, 1987), the entire nerve cord begins to exhibit fictive swimming, not just the stimulated ganglion. Furthermore, stimulation of a single swim gating neuron (cell 204) induces the entire nerve cord to swim (Weeks and Kristan, 1978). The swim CPGs are essentially impossible to uncouple because the swim gating neurons and some members of the CPG have intersegmental-projecting connections, thus the intersegmental coordination is inherent in the CPG circuitry (Brodfuehrer et al., 1995a; Kristan et al., 2005).

Another strategy employed for the coordination of locomotor oscillators is one where a greater degree of flexibility, and often less stereotypy, exists between the oscillators (e.g., Akay et al., 2006). Locomotor behaviors that possess more flexible oscillator coupling can often be paused without a reconfiguration of the body posture or locomotor organs (Stern-Thomlinson et al., 1986; Cacciatore et al., 2000). During these pauses other behaviors can be expressed and upon their completion the locomotor step-cycle can be resumed or restarted. More flexibly coupled systems often require greater control from higher-order CNS regions and/or sensory inputs (Büschges, 2005). Walking in humans is an example of flexibly coupled locomotor system (Yang and Gorassini, 2006). To produce walking, the numerous spinal CPGs that control the

multiple joints, muscle groups and individual appendages (i.e., legs and arms) must be activated in the correct order with the proper timing relationships. Individual steps, however, can be modified mid-cycle to accommodate terrain features or to overlay other behaviors such as kicking a ball. Additionally during normal walking, the arms swing rhythmically, however, humans can easily uncouple the activities of the arms and legs during walking or running behaviors. In cats, the activities of the cervical walking CPGs can entrain those of the lumbar CPGs, but the activities of the fore- and hind-limbs can also be uncoupled (Akay et al., 2006).

In more flexibly coupled oscillator systems the inter-oscillator coordination can be managed by local sensory signals or by longer-distance projecting signals often from higher-order CNS regions (i.e., central signals from the brain) (see below). The relative contributions of sensory or central coupling signals can vary. For example, in the stick insect, although a relatively weaker central coupling of the segmental CPGs has been shown, sensory information is the main source of inter-oscillator coupling (Büschges, 2005; Borgmann et al., 2007; Borgmann et al., 2009). In non-human walking vertebrates in addition to local proprioceptive information (Akay et al., 2006) central mechanisms play a role in coordination of the limbs (Juvin et al., 2005). Although no specific cellular correlates have been elucidated, longer distance propriospinal neurons play a role in the coupling of the anterior and posterior limbs (Juvin et al., 2005). Additionally, projection neurons from the brainstem and midbrain may play a role in interlimb coordination of cats (Shimamura and Kogure, 1983; Drew et al., 1986).

My thesis research has shed light on some of the underlying mechanisms for the coordination of flexibly coupled locomotor oscillators. In Chapter 3, I provide evidence that although the CPGs for crawling in the medicinal leech communicate with each other during fictive crawling, descending information from the cephalic ganglion is necessary

for normal intersegmental coordination during fictive crawling. In Chapter 4, I show that a single neuron, located in the cephalic ganglion, R3b-1, is both necessary and sufficient for the intersegmental coordination of the crawl oscillators. In Chapter 4, I also propose a mechanism for oscillator coupling that requires both R3b-1 signaling as well as local, inter-oscillator coupling.

Higher-order control of locomotion

Above, I provided examples of how higher-order (i.e., command-like) neurons can activate oscillatory neural circuits. Command-like neurons, in addition to activating locomotion, often influence locomotor behaviors in other ways as well (e.g., Buchanan and Cohen, 1982; Böhm and Schilderberger, 1992). One prominent role of these neurons is to gather and integrate information from an animal's environment (i.e., sensory) and internal state (i.e., hunger, reproductive, etc.) and select which behavior(s) to express or not express (Dubuc et al., 2008; Kristan, 2008). Higher-order decision-making systems analyze incoming state information and continually update their 'decisions' as conditions change. This process is extremely complex and can involve cognitive processes such as memory, motivational state and many others, especially in vertebrate animals (Platt, 2002; Pesaran, 2010). My final discussion point will be restricted to the simpler decision of which locomotor behavior to select for expression.

Command-like decision-making neurons can be located in higher-order areas of the CNS such as the brain (or cephalic ganglion) or can be distributed throughout the spinal cord or nerve cord. For example, both the descending cephalic neuron R3b-1 (mentioned above) and the distributed segmental swim-gating neurons 204 are involved in locomotor decision making.

The classical decision-making function of a command-like neuron is to turn on or turn off a given behavior. Kupfermann and Weiss (1978) defined a command neuron as a cell or population of cells that is/are both necessary and sufficient for a given behavior. Although this definition fell under immediate criticism, studies since the inception of this definition have used it as a framework for describing the functional role of command-like neurons in the control of behavior (e.g., Ritzmann et al., 1980; Nolen and Hoy, 1984; Hedwig, 2000). In addition to the command neuron for swimmeret beating in crayfish (Wiersma and Ikeda, 1964) and the 404 neurons of the locust (Pearson, et al., 1985) that I mentioned above, other command neurons have been found for walking in the crayfish (Bowerman and Larimer, 1974) as well as escape movements in both the crayfish (Olson and Krasne, 1981) and teleost fish (Mauthner neurons; Korn and Faber, 2005). In Chapter 4, I determined that R3b-1, in addition to its other numerous functions (see below), is necessary and sufficient for crawling in the medicinal leech.

A fascinating aspect of behavioral selection is seen in command-like neurons that elicit different behaviors based on some context. A number of studies have focused on how sensory context affects behavioral selection. For example, Ritzmann et al. (1980) reported that stimulation of the command-like (i.e., necessary and sufficient) dorsal giant interneuron in the brain of the cockroach elicited running or flying behavior. The specific form of locomotion that was activated was reliably predicted by whether or not the legs were in contact with a substrate. When at least one of the lower legs was touching the substrate, stimulation of the giant interneuron led to running behavior, however, if no legs were touching a substrate the animal would begin to fly. In the medicinal leech, Esch et al. (2002) established that intracellular stimulation of the descending projection neuron, R3b-1, activated either overt crawling or swimming depending on the fluid levels surrounding the body. When the body was submerged in

fluid, R3b-1 stimulation induced swimming and when fluid levels were low it induced crawling.

In addition to sensory context, some neurons will elicit differential responses based on an animal's 'behavioral state'. In the cricket, stimulation of a command-like neuron for singing produces short, incomplete and non-sustained chirps, however, if the animal has sung recently, stimulation of the same neuron leads to robust, sustained and complete chirping. Also in the cricket, activation of the command-like interneuron (Int-2) can cause avoidance steering movements in response to auditory stimuli that resemble bat echolocation pulses, but only during flight (Nolen and Hoy, 1984). When the animal is not flying, stimulation of Int-2 has no overt behavioral effect. Thus the experience or behavioral state of an animal is critical to understanding the behavioral impact of any given command-like neuron or set of cells.

I postulate behavioral state can be represented as a relative increase or decrease in the concentration of a given neuromodulator within a region of the CNS (Selverston, 1985). This is supported in a study of the medicinal leech whereby focused application of serotonin to the cephalic ganglion reduces swimming (Crisp and Mesce, 2003), perhaps by inhibiting higher-order trigger neurons that control swimming (Tr-1, Tr-2 and SIN1) (Crisp and Mesce, 2006). Although Tr-2 and SIN1 are more associated with swim termination, inhibition of these command-like neurons ultimately led to a reduction in the firing of the swim gating neuron 204 and thus a reduction of swimming behavior. Chapter 4 expands the dimensionality of context-sensitivity of R3b-1 in the medicinal leech to include modulatory-state sensitivity. When R3b-1 is stimulated in isolated nerve cord (i.e., fictive) preparations, either fictive crawling or swimming is observed. In the absence of modulators, the form of fictive locomotion expressed remains unchanged upon subsequent bouts of R3b-1 activation (Esch et al., 2002).

When dopamine (DA) is applied to these fictive preparations, only fictive crawling is expressed in response to R3b-1 spiking, regardless of the form of fictive locomotion that was expressed prior to DA application. Thus, R3b-1 responds differentially to both sensory (Esch et al., 2002) *and* modulatory contexts (Chapter 4).

Last, some command-like neurons are involved in controlling multiple aspects of a given behavior. In the cricket, a command-like neuron that can both initiate and terminate walking behavior also serves to modulate the speed of walking movements (Böhm and Schildberger, 1992). In the cat (Mori et al., 1989) and lamprey (Buchanan and Cohen, 1982), varying the stimulation amplitude of reticulospinal neurons (which also activate locomotion) modulates the speed of walking and fictive swimming, respectively. In the medicinal leech, the stimulus amplitude of the command-like swim gating neuron (cell 204) is negatively correlated with fictive swimming frequency (Weeks and Kristan, 1978). R3b-1 spiking during fictive crawling is positively correlated with its frequency (Chapter 4). In addition to activating and modulating the speed of fictive crawling, R3b-1 acts with local inter-oscillator signaling to regulate the intersegmental coordination of the relatively independent crawl CPGs (Chapter 4). The role R3b-1 plays in coordinating the CPGs for crawling adds an exciting novel dimension of influence for command-like neurons in generating behaviors. The collective investigations of R3b-1 have determined that this neuron is truly a unique interneuron for controlling locomotion (Esch et al., 2002; Chapter 4). To my knowledge, no other single neuron has been implicated in as many dimensions of a well defined behavior.

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Figure 1-1: Schematic depicting the motor control infrastructure.

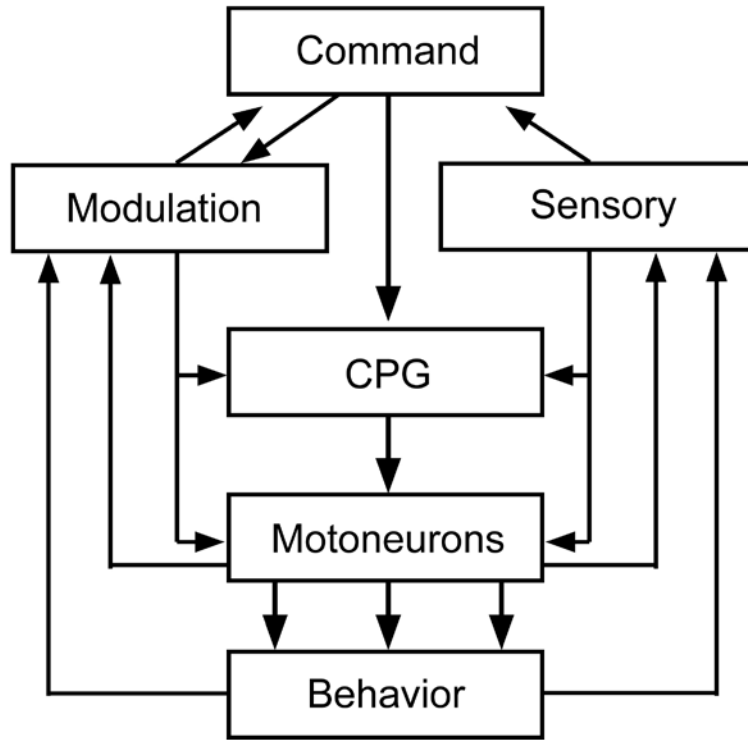


Figure 1-1: Schematic depicting the motor control infrastructure.

Chapter Two:

Dopamine Activates the Motor Pattern for Crawling in the Medicinal Leech

Puhl JG, Mesce KA (2008) Dopamine activates the motor pattern for crawling in the medicinal leech. J Neurosci 28:4192-4200.

Locomotion in segmented animals is thought to be based on the coupling of “unit burst generators”, but the biological nature of the unit burst generator has been revealed in only a few animal systems. We determined that dopamine (DA), a universal modulator of motor activity, is sufficient to activate fictive crawling in the medicinal leech, and can exert its actions within the smallest division of the animal’s CNS, the segmental ganglion. In the entire isolated nerve cord or in the single ganglion, DA induced slow anti-phasic bursting (ca. 15-second period) of motoneurons known to participate in the two-step elongation-contraction cycle underlying crawling behavior. During each cycle, the dorsal (DE-3) and ventral (VE-4) longitudinal excitor motoneurons fired roughly 180° out of phase from the ventrolateral circular excitor motoneuron (CV), which marks the elongation phase. In many isolated whole nerve cords, DE-3 bursting progressed in an anterior to posterior direction with intersegmental phase delays appropriate for crawling. In the single ganglion, the dorsal (DI-1) and ventral (VI-2) inhibitory longitudinal motoneurons fired out of phase with each DE-3 burst, further confirming that the crawl unit burst generator exists in the single ganglion. All isolated ganglia of the CNS were competent to produce DA-induced robust fictive crawling, which typically lasted uninterrupted for 5-15 minutes. A quantitative analysis indicated that DA-induced crawling was not significantly different from electrically-evoked or spontaneous crawling. We conclude that DA is sufficient to activate the full crawl motor program and that the kernel for crawling resides within each segmental ganglion.

Introduction

A major challenge in the field of locomotion is to understand the timing and coordination of body movements that enable an animal to traverse smoothly through its environment. Although sensory feedback is undoubtedly important (Pearson, 2000), the neural basis of locomotion is typically founded on the operation of a central pattern generator (CPG), whereby the central nervous system (CNS) generates the timing elements and phase relationships of the movements expressed. Much is known about the neural underpinnings of CPGs in general (Marder and Calabrese, 1996), but far less is understood about pattern generators or the putative “unit burst generators” (Grillner, 1981) that control locomotion in segmented animals. Studies of the rodent spinal cord, lamprey and additional lower vertebrates (e.g., zebrafish and tadpoles), have contributed significantly to the elucidation of circuitry underlying locomotor control (Grillner, 2006). Invertebrate systems have also been immensely instructive. Examples include swimmeret beating in the crayfish (Mulloney and Hall, 2007), insect flight (Robertson and Pearson, 1985) and swimming in the leech (Brodfuehrer et al., 1995a). Aside from swimmeret beating (Murchison et al., 1993), the kernel for rhythmic movements is often distributed across segmental units (e.g., flight circuit; Robertson and Pearson, 1983) or is difficult to elicit and sustain in a single segmental unit. In the leech CNS, for example, serotonin can promote the semblance of the swim motor pattern in a single isolated ganglion, but it is weak and not sustained over time (Hocker et al., 2000). This response contrasts sharply with the robust swimming achieved when a chain of ganglia is stimulated (chemically or electrically) or when intersegmental swim-gating neurons are present (Weeks, 1981).

Ideally, an animal preparation that is competent to produce a reliable, robust and sustained locomotor rhythm in a single segmental unit would greatly simplify the

elucidation of its rhythmogenesis, and serve as a model to understand why some kernels are distributed and some are not. It also could provide a unique system to study intersegmental coordination, as each segmental oscillator would have the capacity for complete independence.

Here, we asked whether the kernel for vermiform crawling was confined to a single segmental division of the CNS. Crawling is a prominent form of locomotion in the leech and consists of the animal releasing its anterior sucker, elongating its body while staying close to the substrate, attaching its anterior sucker, releasing its posterior sucker, and then contracting its entire body with re-attachment of the posterior sucker (Gray et al., 1938; Stern-Tomlinson et al., 1986). This cycle is repeated and acts to propel the leech forward. In addition, fictive crawling has been defined in detail (Eisenhart et al., 2000), and key motoneurons have been identified (Baader, 1997).

In this report, we established how best to activate the crawl CPG in its entirety. We tested whether dopamine (DA), an important neuromodulator of movement, was sufficient to activate crawling. In response to DA application, we describe patterned neural activity obtained from identified motoneurons in preparations that had the entire CNS intact, but isolated from the body. We also examined whether DA could induce fictive crawling in as little as a single isolated ganglion.

Materials and Methods

Animals

Adult medicinal leeches (species: *Hirudo medicinalis* and *H. verbana*) were obtained from Leeches USA (Westbury, NY, USA) and maintained at room temperature in either Buhl spring water (Buhl, MN, USA) or distilled water containing *Hirudo*-salt (0.5 g/L; Leeches USA). Leeches used ranged in size between 2.0-3.5g. Leeches were

anaesthetized on ice or in ice-chilled saline for a minimum of 15 minutes (min) before dissection.

Terminology

The leech nervous system consists of a compound cephalic ganglion (i.e., “head-brain” that comprises the supra- and subesophageal ganglia), the 21 unfused segmental ganglia (designated M1-M21), and a compound terminal ganglion (schematic Fig. 2-1A). In this study, the term “whole nerve cord” refers to a complete CNS preparation dissected from its body.

Each segmental ganglion has a dorsal and ventral layer of neuronal somata, and uniquely identified cells often have a functional as well as numerical designator (Fig. 2-2B,C). For example, cell 3 and cell 4 are known, respectively, as the dorsal and ventral longitudinal excitor motoneurons (DE-3 and VE-4). Cells 1 and 2 are also referred to as the dorsal and ventral longitudinal inhibitor motoneurons (DI-1 and VI-2). DE-3, which was used as a crawl-burst monitor cell, extends its axon through the dorsal posterior (DP) nerve root.

CNS preparations

After exposing the intact nerve cord, the surrounding sinus was cut dorsally along its entire length and removed from the connectives and ganglia prior to intracellular recording. The glial sheath was left intact on all preparations recorded. Typically, four DP nerves were left attached to the middle body ganglia (M7-M15) and excess tissue around the nerve root was removed. The CNS was placed into chilled normal saline during dissection and prior to application of dopamine. Normal saline contained (in mM): 116.0 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.5 MgCl₂, 10.0 dextrose, 10.0 Trizma pre-set crystals, pH 7.4 (Sigma-Aldrich, St Louis, MO, USA) (adapted from Nicholls and Baylor, 1968).

Single ganglia were removed from adjacent anterior and posterior ganglia by transecting all connectives; one or both of the DP nerves were left attached.

Electrophysiological recordings and cell identification

To monitor fictive locomotor behaviors, we recorded extracellular unit activity from a given DP nerve using a suction electrode. The signal was amplified using a Grass P15 AC pre-amplifier (Grass Technologies; West Warwick, RI, USA) and recordings were digitized using a MacLab/4s data acquisition system (ADInstruments, NSW Australia) attached to a Macintosh personal computer (Apple, Cupertino, CA, USA). Fictive crawling was identified by monitoring the activity of DE-3, which was the largest spike in the DP recording (Kristan et al., 1974). Fictive crawling was defined as three or more consecutive bursts of DE-3 with a period ranging between 5-25 seconds (s) (Eisenhart et al., 2000). For the calculation of intersegmental burst delays, two extracellular DP-recording electrodes were interposed by 1-5 ganglia. In some preparations, for example, DP nerves from M9 and M14 or M13 and M16 were recorded, whereas in other preparations, more anterior ganglia (M7 and M9) were recorded. These various recording sites ensured that the intersegmental delays calculated represent a reliable measure of delay constancy.

The intracellular activity of identified neurons was recorded using a Dagan IX2-700 amplifier (Dagan Corp, Minneapolis, MN, USA) and was digitized as described above. Intracellular electrodes had resistances of 40-65 M Ω and were filled with a solution of 2 M potassium acetate and 20 mM potassium chloride. Intracellular electrode tips were typically back filled with 5% (w/v) Neurobiotin (Vector Laboratories, Burlingame, CA, USA) dissolved in a 2 M potassium acetate solution for cell labeling.

All neurons were identified by the stereotypical position and size of their somata, and their electrophysiological signature. Cell identities were further confirmed by iontophoretically injecting 5% Neurobiotin (+0.5-1 nA) into a given cell for at least 10 min. Tissue was subsequently fixed, processed, treated with Cy3-conjugated Streptavidin (1:50 dilution in PBS for 18-24 hours at 4°C; Jackson ImmunoResearch, West Grove, PA, USA), and prepared for viewing according to the methods of Gilchrist et al. (1995).

Dopamine superfusion

Dopamine-containing saline was typically delivered into the recording chamber by gravity with a flow rate of 1-2 mL/min. Outflow was maintained by passive siphon to preserve fluid levels at 2-3 mL. For some experiments, electric peristalsis pumps were used to introduce and remove the DA solutions. A 1 mM DA-containing saline solution was made fresh daily, stored in the dark at 4° C and used within 4 hours. Immediately prior to use, a portion of the DA concentrate was diluted in normal saline. DA-containing saline was passively warmed to room temperature prior to superfusion.

Determining effective DA concentrations

To determine which concentrations of DA were most effective for inducing fictive crawling, DA of varying concentrations was superfused onto intact CNS and isolated ganglion preparations. We used DA concentrations (in μM) of 10, 50, 75, 100, 200 and 500. The delivery rate of DA was held at 1-2 mL/min for a minimum of 30 min. If we did not observe crawling, as strictly defined above, we considered a given concentration to be ineffective. The degree of effectiveness was determined by the percentage of preparations that exhibited fictive crawling during DA superfusion.

Data analysis and statistical methods

The period of each crawl cycle was determined by calculating the time between midpoints of consecutive DE-3 bursts. The burst mid-point was calculated by adding the burst onset and 0.5 times the burst duration. The mean crawl period for all DA-induced fictive crawling was calculated by analyzing 10 consecutive crawl cycles. Mean crawl periods for spontaneous or electrically-induced fictive crawling were calculated by analyzing 4-10 crawl cycles. This mean comprised a single sample point in the statistical analyses (i.e., a sample size of $n = 10$, represented 100 DE-3 bursts). Calculations of crawl periods extracted from published recordings and from spontaneous fictive crawling consisted of 3-6 periods per sample point. The duty cycle was calculated by dividing the DE-3 burst duration by the coinciding crawl period for that burst.

Parameters of the crawl rhythm described above were compared across three fictive crawl groups. The groups consisted of intact CNS preparations in which crawling was 1) spontaneous or electrically-induced or 2) DA induced. The third group consisted of crawling in a single, isolated ganglion induced by DA. We calculated the elongation duration, contraction duration and period for overt crawling in intact leeches. Crawling leeches were video recorded using a Sony DCR-TRV840 camcorder (Sony, New York, NY, USA). The video was digitized onto a personal computer using Microsoft Moviemaker (Microsoft, Redmond, WA, USA). The video was analyzed frame-by-frame to determine the parameters of crawling. Release and reattachment of the head sucker denoted the onset and end of elongation. Release and reattachment of the posterior sucker denoted the onset and the end of contraction. The period of overt crawling was calculated by determining the duration between the onset of elongation and the end of contraction.

All means presented are \pm the standard error of the mean (SEM). Two-tailed, one-way ANOVA tests, Student's *t*-tests and linear regressions were performed using the software package R (R Development Core Team, 2007; URL: <http://www.R-project.org>). The confidence level used was 0.95 ($\alpha = 0.05$). Linear regressions were performed using the least-squared method.

Results

Dopamine is sufficient to activate fictive crawling in isolated, intact CNS preparations

A crawling-like motor rhythm was observed in the DP nerves of 19 out of 21 intact CNS preparations that were superfused with 75-100 μ M DA (Fig. 2-1C). Other concentrations of DA were tested, but we found 75 μ M to be optimal for inducing this crawl-like rhythm. In 12 of these 19 preparations (63 %), the crawl-like burst activity propagated through the nerve cord in an anterior-to-posterior direction, similar to fictive crawling previously reported (Eisenhart et al., 2000; Fig. 2-1C). In the remaining 7 of 19 nerve cords, the DA-induced crawl-like bursts appeared uncoordinated among segments. Of the 12 preparations showing intersegmental burst coordination, 5 exhibited the anterior-to-posterior propagation during the period of DA application, whereas the other 7 showed this propagation only after DA had begun to be washed out of the CNS. Two preparations expressed appropriate propagation during DA application, which eventually subsided, but later resumed during DA washout.

As shown in figure 2-1B, DP nerve recordings were quiescent prior to the application of DA, and the annulus erector (AE) motoneuron ($n = 4$) fired tonically. After approximately 3 min, crawl-like episodes emerged as indicated by bursting of the largest unit recorded in the DP nerve, the dorsal longitudinal excitor motoneuron (DE-3). Several minutes later, these crawl-like bursts propagated in an anterior-to-posterior

direction along the nerve cord (Fig. 2-1C). The intersegmental delay (ISD) between the onset of crawl-like bursts induced during DA superfusion was 0.64 ± 0.09 s per segment ($n = 5$). In nerve cords that exhibited anterior-to-posterior propagation only during DA washout ($n = 7$), and during periods of DA application and washout ($n = 2$), the ISD was 0.44 ± 0.08 s per segment ($n = 9$). Both ISDs were within the range (0.35-1.15 s/segment) we calculated using data of spontaneous crawling obtained from Eisenhart et al., 2000 and Briggman and Kristan, 2006.

To determine whether the DA-induced pattern of activity we obtained, indeed, met the established criteria for fictive crawling (Eisenhart et al., 2000), we recorded from motoneurons known to participate in crawling movements (Baader, 1997). A schematic of the experimental preparation, placement of electrodes, and location of somata studied are depicted in figure 2-2A-C. Using intracellular methods we recorded from longitudinal and circular motoneurons during DA-induced crawl-like activity. These cells were previously reported to oscillate in phase with either the elongation or contraction steps of fictive crawling (Eisenhart et al., 2000, Baader, 1997). Intracellular recordings indicated that the activity of the ventrolateral circular excitor (CV) motoneuron, activated during the elongation step, oscillated approximately 180° out of phase with DE-3 (recorded in the DP nerve), which was active during the contraction step ($n = 5$). CV was hyperpolarized approximately 2-4 mV and its action potentials were largely suppressed during DE-3 bursting (Fig. 2-2D). Because the dorsal and ventral longitudinal muscles are co-activated during each contraction step, in contrast to swimming when they are in anti-phase, we wanted to establish that the ventral longitudinal excitor motoneurons (e.g. VE-4) fired in-phase with DE-3. During DE-3 bursts, VE-4 was depolarized and fired bursts of action potentials that were in-phase with DE-3 ($n = 4$; Fig. 2-2E). There was a $93.97\% \pm 4.95\%$ overlap between DE-3 bursts and maximal depolarization of VE-4. The out-of-

phase activity patterns of the circular and longitudinal motoneurons (CV and DE-3), coupled with the in-phase activity patterns of the longitudinal excitator motoneurons (DE-3 and VE-4) is consistent with previous data that were used to define the crawling motor pattern (Eisenhart et al., 2000).

The fact that DA (in the absence of additional modulators) can activate a fictive locomotor behavior in the dissected CNS is significant, yet it begs the question of where the DA-sensitive pattern generating circuitry resides. Does the pattern-generating kernel reside in a single segmental unit or is it distributed among more than one segmental division? To investigate this question, we conducted experiments using the smallest division of the CNS, the segmental ganglion.

Dopamine activates the crawling motor pattern in an isolated, single ganglion

We observed fictive crawling-like behavior in the isolated, single ganglion after administration of DA (Figs. 2-3 and 2-4). This rhythm was observed in 95 out of 122 (78%) preparations that were superfused with 75-100 μ M DA. Ganglia from the middle body segments (M7-M15; n = 112), and from anterior (M2-M4; n = 6) and posterior (M16-M21; n = 7) body regions (see below) were all capable of reliably producing fictive crawl-like behavior. The quantitative analysis of this locomotor pattern, however, was primarily conducted on ganglia in the middle body segments M7-M15.

To determine whether the DA-induced pattern of activity in the single ganglion met the established criteria for fictive crawling (aside from intersegmental coordination), we recorded from an ensemble of motoneurons known to define fictive crawling (Baader, 1997; Eisenhart et al., 2000). We analyzed the rhythm of the excitatory longitudinal motoneurons DE-3 and VE-4, the circular motoneuron CV, and the inhibitory longitudinal motoneurons DI-1 and VI-2. In particular, we established that the timing and phase

relationships among these motoneurons were indistinguishable from fictive crawling that occurred spontaneously or was electrically-induced.

Figure 2-3A shows an intracellular and extracellular recording of DE-3 during superfusion of 75 μ M DA ($n = 5$). DE-3 exhibited a 12-16 mV depolarization with 1-3 mV spikes at the peak of each slow-wave. When DE-3 spiking subsided, the membrane potential repolarized to baseline levels that were consistent over time. Individual DE-3 spikes observed in the intracellular recording were correlated with the largest spike in the DP nerve (Fig. 2-3A, shaded areas). This 1-to-1 relationship re-confirmed that the largest spikes observed in the DP nerve recordings were those of DE-3 and, furthermore, that DE-3 was correctly identified. As previously presented in the intact CNS (see above), VE-4 was active in phase with DE-3 ($n = 6$). In the single ganglion, this cell exhibited a slow depolarization of 10-12 mV with 1-3 mV spikes at the peak of each slow-wave (Fig. 2-3B). At peak depolarization, VE-4 was clearly in phase with each burst of DE-3 (Fig. 2-3B). In contrast, CV excitation, which gives rise to elongation, fired rhythmically out of phase with both DE-3 and VE-4 ($n = 6$; Fig. 2-3C). CV peak hyperpolarizations of 2-3 mV were always in phase with the mid-points of the DE-3 bursts. Spikes in CV of 4-6 mV were routinely observed during periods of DE-3 quiescence.

Based on the circular and longitudinal motoneurons being out of phase and the dorsal and ventral excitatory longitudinal motoneurons firing in-phase, we suspected that DA was, indeed, activating fictive crawling in the single ganglion. To strengthen our support for such a conclusion, we investigated yet another class of motoneurons – the inhibitory longitudinal motoneurons. These motoneurons are activated to prevent the co-contraction of the CV antagonist muscle groups, i.e., longitudinal muscles (excited by DE-3 and VE-4) (Stuart, 1970). Figure 2-4A,B depicts the activities of the inhibitory

longitudinal motoneurons DI-1 and VI-2, which fired rhythmically and were phase-locked with step-cycles of the crawl rhythm. Peak excitation of DI-1 correlated with the termination of DE-3 bursts ($n = 4$). This activity is consistent with DI-1 inhibiting the dorsal longitudinal muscle, i.e., the muscle target of DE-3, thus preventing contraction of the body (Fig. 2-4A). The motoneuron VI-2, which inhibits the ventral longitudinal muscle target of VE-4, was also rhythmically active and phase-locked with the crawl rhythm ($n = 4$). VI-2 fired action potentials that were out of phase with VE-4 and its maximum hyperpolarization occurred during each DE-3 burst (Fig. 2-4B).

Last, to document that the far anterior and posterior ganglia were also capable of producing DA-induced crawling, we recorded from DP nerves of single isolated ganglia taken from these body regions. For example, M2 (Fig. 2-5A) and M19 (Fig. 2-5B) exhibited DA-induced ($100 \mu\text{M}$) crawling similar to that obtained for the middle body ganglia.

Analysis of specific features of dopamine-induced fictive crawling in the single isolated ganglion

In addition to analyzing the DA-induced rhythm, we measured the effect of varying DA concentrations, the latency to the onset of fictive crawling and the duration of single bouts of uninterrupted fictive crawling. Figure 2-6A shows the percentage of preparations that produced fictive crawling at 0, 10, 50, 75, 100, 200 and $500 \mu\text{M}$ DA. From this, we determined that DA concentrations of 75-100 μM were optimal, activating the rhythm in 78% of preparations ($n = 122$). Slightly lower and higher DA concentrations were able to activate fictive crawling, but were clearly less effective. In particular, preparations exposed to higher DA concentrations showed an initial increase

in the tonic firing of DE-3 (monitored in the DP nerve) for 2-5 min, followed by a prolonged quiescence of DE-3 activity and that of all other units in the DP nerve.

Figure 2-6B shows the latency to onset for DA-induced fictive crawling from 19 randomly selected experiments that were treated with 75-100 μM DA. While the majority of single ganglia exhibited fictive crawling in less than 6 min, some preparations required over 9 min. The average latency to onset was 6.0 ± 2.3 min which ranged from 0.7 - 18.3 min ($n = 19$). The majority of DP nerves in DA-treated preparations exhibited a period of quiescence lasting approximately 30-60 s prior to the onset of rhythmic activity. As rhythmic activity emerged, DE-3 bursting, in some preparations, exhibited irregular periods and duty cycles that eventually stabilized, typically within 0.5-1 min.

In addition to the latency to onset of fictive crawling, we calculated the duration of uninterrupted bouts of DA-induced (75-100 μM) fictive crawling in single isolated ganglia ($n = 20$) (Fig. 2-6C). The average duration of a single episode was 12.7 ± 2.6 min, with two crawl episodes lasting over 20 min. Application of higher DA concentrations (200-500 μM) resulted in relatively short (< 3 min) latencies to crawl onset, and the duration of uninterrupted crawl bouts was significantly shorter (3.5 ± 1.8 min, $n = 3$ of 8 ganglia that crawled; Student's t -test $p < 0.012$). There were, however, no obvious differences in the period and duty cycle of the ensuing crawl rhythms induced at higher concentrations.

The dopamine-induced fictive crawl rhythm is statistically indistinguishable from spontaneous or electrically-induced fictive crawling

To establish whether the DA-induced fictive crawling we obtained was indistinguishable from fictive crawling that was spontaneous or electrically induced, we measured the period, DE-3 burst duration and duty cycle of the rhythm, and compared these data to published results and data from our experiments. A prominent feature that

differentiates the crawl rhythm from other locomotor rhythms, such as swimming, is the period of the DE-3 bursts (Kristan et al., 2005). For the majority of our analyses, we measured the period by calculating the time between burst mid-points (see Methods). The burst mid-point was used (in contrast to burst onset) to help reduce bias or error when calculating the beginning and ending of DE-3 bursts, although they were usually obvious. Three fictive crawling groups were analyzed and compared: spontaneous or electrically-induced fictive crawling (Non-DA whole cord), DA-induced fictive crawling in intact CNS preparations (DA whole cord) and DA-induced fictive crawling in single, isolated ganglia (DA single ganglion). Figure 2-7A shows the mean period for each fictive crawling group and for overt crawling in intact leeches. The average period for the non-DA whole cord group was 19.3 ± 1.8 s. The average periods for the DA whole cord and the DA single ganglion groups were 14.3 ± 1.7 s and 15.5 ± 1.9 s respectively. There were no significant differences between the three groups (one-way ANOVA, $p = 0.14$). The period of overt crawling in intact leeches was 3.3 ± 0.2 s. This was significantly different (Student's t -test, $p < 0.001$) from the fictive crawling groups and coincides with published results (Eisenhart et al., 2000).

In addition to the period, we compared the duration of DE-3 bursts and the duty cycle across the three experimental groups (described above). The average DE-3 burst durations for the non-DA whole cord, DA whole cord, and DA single ganglion groups were 5.5 ± 0.9 s, 5.1 ± 0.6 s and 5.4 ± 0.6 s respectively (Fig. 2-7B). There were no significant differences between the three groups (one-way ANOVA, $p > 0.5$). We also calculated the DE-3 duty cycle (ratio of DE-3 burst duration to the coinciding period), because it preserves the relationship of an individual DE-3 burst with its period and not the mean period. The duty cycles for the non-DA whole cord, DA whole cord, and DA single ganglion groups were 0.39 ± 0.04 s, 0.39 ± 0.03 and 0.34 ± 0.02 respectively (Fig.

2-7C). No significant differences existed between these groups (one-way ANOVA, $p = 0.45$). To compare the duty cycle of fictive crawling to overt crawling, we calculated the ratio of contraction time to the period of overt crawling in intact crawling leeches. We named this ratio the “contraction duty cycle” because DE-3 equates with the contraction step-cycle. The contraction duty cycle for overt crawling was 0.30 ± 0.01 (Fig. 2-7C). No significant differences were found between the duty cycles for the four groups (ANOVA, $p > 0.1$).

Although the average duty cycles for the three fictive and the overt crawling groups were not significantly different, the question remained whether the duty cycle was maintained throughout the range of crawl periods for the DA-induced fictive crawling groups. To investigate this, we performed a linear regression analysis to determine if the period of a given DA-induced crawl cycle could predict the duration of the coinciding DE-3 burst. Figure 2-8A-C shows scatter plots of the mean crawl period versus DE-3 burst duration for each fictive crawl group, with resulting linear regression lines. For comparison, Fig. 2-8D shows the same information for overt crawling. Data from the regression analysis determined that throughout the range of periods for fictive crawling (5-25 s) (Eisenhart et al., 2000), a linear relationship exists between the crawl period and the DE-3 burst duration (or contraction duration) for DA-induced fictive crawling and for overt crawling. This relationship, however, was strongest for the DA single ganglion and overt crawl groups (Figs. 2-8C and 2-8D).

Discussion

In this study, we determined that DA activates the motor program for crawling in as little as a single isolated ganglion of the medicinal leech. This is the first report of a singular biogenic amine being sufficient to activate a robust, reliable, and sustained

locomotor rhythm in one division of a segmented animal. DA was also found to activate fictive crawling in the isolated whole nerve cord, where appropriate intersegmental coordination between segments could be observed. Based on data obtained in the single ganglion, we can conclude that the rhythm-generating circuitry for crawling exists within the smallest segmental division of the leech CNS, thus empirically defining the unit burst generator for crawling. In addition, because each body segment contains one unique pair of DA-containing neurons, which richly innervates each segmental ganglion or neuromere (Crisp et al., 2002; Crisp and Mesce, 2004), the release of DA *via* these neurons probably provides the natural route for DA's putative actions on the crawl CPG.

Previous studies determined that there was not a significant correlation between the duration of DE-3 bursts and the period for fictive crawling in isolated CNS preparations (Eisenhart et al., 2000). Our study provides evidence that a linear relationship exists between the duration of DE-3 bursts and the period for fictive crawling induced by DA in the isolated single ganglion (Fig. 2-8C). A similar correlation also exists for overt crawling, although the periods were shorter (Fig. 2-8D). These results suggest that the CPG for crawling possesses intrinsic timing parameters that produce a basic crawling rhythm when unperturbed by outside factors, such as sensory inputs or information from adjacent segments.

Comparisons between crawling and swimming in the leech

Crawling and swimming are the two most prominent forms of locomotion in the leech, but the mechanisms underlying swimming have received comparatively more attention. Swimming has been thoroughly described at the level of its pattern-generating neural networks (Brodfuehrer and Thorogood, 2001, Brodfuehrer et al., 1995a), gating cells (Kristan and Weeks, 1983), descending command-like cells (Brodfuehrer and

Friesen, 1986, O’Gara and Friesen, 1995, Brodfuehrer et al., 1995b; Esch et al., 2002) and modulation by serotonin (Nusbaum and Kristan, 1986; Crisp and Mesce, 2006). Insights into the neuronal bases of both these locomotor patterns can now be advanced by comparing their organization and regulation.

A study by Crisp and Mesce (2004) reported that DA inhibits fictive swimming but not crawling behavior, first suggesting that DA may promote crawling. Because swimming and crawling appear to share rhythm-generating neurons (Esch et al., 2002; Briggman and Kristan, 2006), it may be necessary for DA to suppress components of the swim circuit so that crawling can emerge. Our study indicates that the crawl CPGs, which are the targets of DA modulation, are present in ganglia located in the anterior, middle and posterior segments of the leech. These results contrast with the segmental nature of the swim CPGs, which appear to be distributed in a more heterogeneous manner. For example, Hocker et al. (2000) reported that short episodes of fictive swimming could be obtained in a fully isolated ganglion taken from middle-body regions, but not so easily from a ganglion residing in anterior body regions. A ganglion from posterior body segments was incompetent to produce any fictive swimming in isolation.

In addition to differences in the competency of anterior vs. posterior isolated ganglia to exhibit swim activity, adjacent ganglia and intersegmental neurons are necessary for sustained and robust swimming, in contrast to crawling. Weeks (1981) determined that robust fictive swimming could be induced in nearly isolated ganglia when the two (lateral) connectives were severed, leaving only the small (medial) Faivre’s nerve (FN) between adjacent ganglia. The FN contains the axons of the swim gating neurons, cells 204/205, that when depolarized, provide excitatory drive to the swim oscillators (Weeks, 1982a, Weeks, 1982b), activate all other swim-gating neurons, and activate fictive swimming in all FN-connected ganglia for a given length of CNS (Weeks

and Kristan, 1978). These data show that although certain ganglia possess a complete oscillator for swimming, these oscillators must rely on excitatory drive from local and adjacent segments for their activation, even in the presence of serotonin. Our study demonstrates that the unit oscillators for crawling are competent to generate crawling in anterior through posterior segmental divisions of the CNS and, upon treatment with DA, CPG activation can be sustained without the need for intersegmental drive.

Such organizational differences between swimming and crawling mirror the contrasting nature of these two behaviors. Swimming is a much faster locomotor behavior (0.5-2 Hz), and consists of anti-phasic dorsal-ventral undulations that propagate along the flattened body with an anterior-to-posterior phase progression, essentially forming a sinusoidal wave that propels the animal forward through its fluid environment. Importantly, the entire length of body must be tightly coordinated throughout each swim cycle to maintain a 3-phase sinusoidal-like progression of 0°, 120°, and 240° (Zheng et al., 2007, Brodfuehrer et al., 1995a). In contrast, during crawling, pauses are commonly observed, especially during the elongation step (Cacciatore et al., 2000; Eisenhart et al., 2000). These pauses are routinely overlaid by other behaviors such as searching movements with the head and whole-body bending (Cacciatore et al., 2000; Mesce et al., 2008; Puhl and Mesce, personal observations). At the conclusion of these sub-routines, crawling is often reinitiated during the same step of the crawl cycle prior to the pause. In order for the leech to suspend and reinitiate crawling, the crawl unit oscillators and the coordination among them must be organized differently from those of swimming. This difference likely accommodates segmental independence and flexibility, which probably accounts for our experimental results. The inherent flexibility of crawling behavior, and its two-step (contraction-elongation)

locomotor cycle, may thus provide a useful cellular model for understanding adaptive and more complex forms of locomotion in vertebrates (Pearson, 2000; Grillner, 2006).

Comparisons with lower vertebrate locomotion

Locomotion in many segmented animals share several key features. Typically, locomotor behaviors can be divided into two phases, the power and return strokes (Büschges, 2005). These two phases, generated by the anti-phasic activation of opposing, or antagonist, motor pools within the same segment comprise a “unit burst generator” (UBG) (Grillner, 1981). These UBGs are iterated to control each segment of the body or locomotor organ and are coordinated by intersegmental connections (Hill et al., 2003; MacKay-Lyons, 2002; Sigvardt and Miller, 1998). These basic features, which are generated centrally, are found in walking animals such as the stick insect (Büschges, 2005), and in swimming animals such as the *Xenopus* tadpole and lamprey (Grillner, 2006).

Based on our data, each segmental ganglion of the leech possesses a complete UBG for crawling that includes both excitatory (Fig. 2-3) and inhibitory (Fig. 2-4) motor outputs. The two phases of the UBG produce the elongation (power stroke) and contraction (return stroke) steps of the crawl cycle. How these UBGs are coordinated across segments is not yet understood, but our preliminary data indicate that DA-treated nerve cords will not display appropriate intersegmental coordination if the compound cephalic ganglion is removed (see Chapter 3). Furthermore, the cephalic nervous system appears to be susceptible to declining fluctuations in DA concentrations, explaining why 7 of 12 whole nerve cords expressed coordinated (intersegmental) crawling only during the period of DA washout. These results mirror those of another study, which showed that sustained and robust fictive swimming was activated when

serotonin was washed from the compound cephalic ganglion after it had been focally applied (Crisp and Mesce, 2003).

Dopamine is involved in controlling locomotion across animal systems

The ability of DA to activate locomotion in the leech and other animals supports the idea that DA is part of a conserved and natural route for the activation and regulation of locomotion. In the planarian flat worm, for example, DA is critical for the proper expression of locomotion (Nishimura et al., 2007). In fruit flies that lack the ability to synthesize DA, locomotion is reduced or abolished (Pendleton et al., 2002). In cases where DA release has been induced, for example, in the newt (Matsunaga et al., 2004) and lamprey (Svensson et al., 2003), locomotion is altered. In the larval zebrafish, the blockade of DA receptors (using clozapine) reduces overall locomotion (Boehmler et al., 2007).

In mammals, DA can induce slow locomotor-like rhythms, for example, as in the lumbar regions of isolated neonatal rat spinal cords (Barriere et al, 2004). This property of DA that induces slow and rhythmic locomotor-related bursting parallels our observations in the leech. To achieve a behaviorally relevant locomotor pattern in rodents, however, a cocktail of neuroactive substances often needs to be administered (Whelan et al., 2000).

The notion that amines can affect CPGs directly is supported by studies in the lamprey where serotonin acts directly on interneurons of the swimming CPG during glutamate-induced rhythmogenesis (Wallen et al., 1989). Although a number of studies support the idea that DA significantly contributes to locomotor control (Svensson, 2003), it has been unclear, to date, if DA activates a locomotor CPG directly. Our current study provides supporting evidence that DA can, indeed, activate the CPG for crawling in the

smallest segmental unit possible, i.e., the single ganglion housing the unit burst generator for crawling. Because locomotion in the leech possesses many of the basic features of vertebrate locomotion, it provides a viable and accessible system to understand conserved mechanisms for locomotor pattern generation, intersegmental coordination and modulation by DA.

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Figures

Figure 2-1. Dopamine-induced fictive crawling in a whole (intact) but isolated CNS. **A:** Schematic of the leech CNS, showing the whole nerve cord and placement of intracellular (small icon) and extracellular (large icon) recording electrodes. Diagonal hatch marks indicate where some of the 21 iterated segmental ganglia are not shown, so as to limit the overall size of the diagram. **B:** Intracellular recording (top) of the annulus erector (AE) motoneuron before treatment with DA. Bottom traces are extracellular dorsal posterior (DP) nerve root recordings from ganglia M7 and M9. **C:** Intracellular recording (top) of the AE motoneuron after superfusion of 75 μ M DA. Bottom traces are extracellular DP nerve root recordings from ganglia M7 and M9. Scale bars in (**C**) apply to (**B**). Numbers in parentheses denote the segment number.

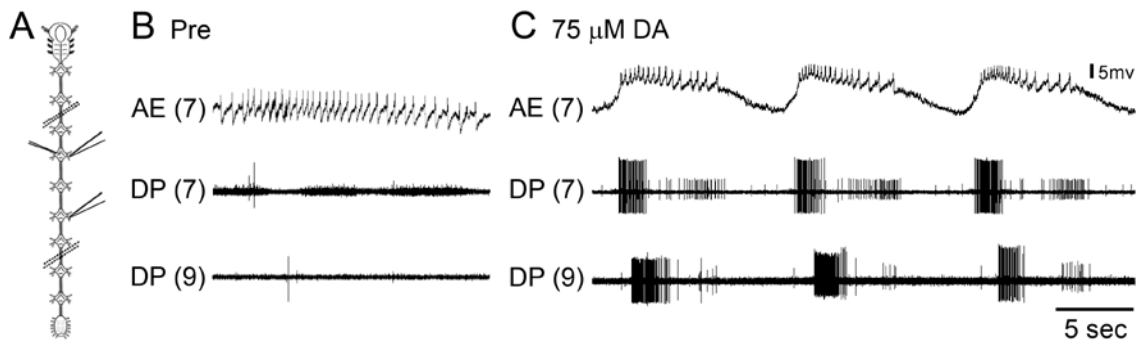


Figure 2-1: Dopamine-induced fictive crawling in a whole CNS.

Figure 2-2. Activities of select motoneurons during dopamine-induced fictive crawling in a whole but isolated CNS. **A:** Schematic of the CNS of a leech for reference and placement of intracellular (small icon) and extracellular (large icon) recording electrodes. **B:** Schematic of the ventral surface of a typical midbody ganglion, showing placement of the AE motoneurons and the CV motoneurons (CV filled in with gray). T, P, N and R (Retzius) somata are common landmarks. **C:** Dorsal surface of a typical midbody ganglion, showing the location of the excitatory and inhibitory motoneurons recorded. **D,E:** Fictive crawling behavior during 75 μ M DA superfusion. Top traces are intracellular recordings of the ventrolateral circular excitor motoneuron (CV) (**D**) and the ventral longitudinal excitor motoneuron (VE-4) (**E**). Bottom traces are extracellular DP nerve root recordings from the same ganglion in which the intracellular recordings were obtained. The largest unit in the DP nerve recordings belong to motoneuron DE-3.

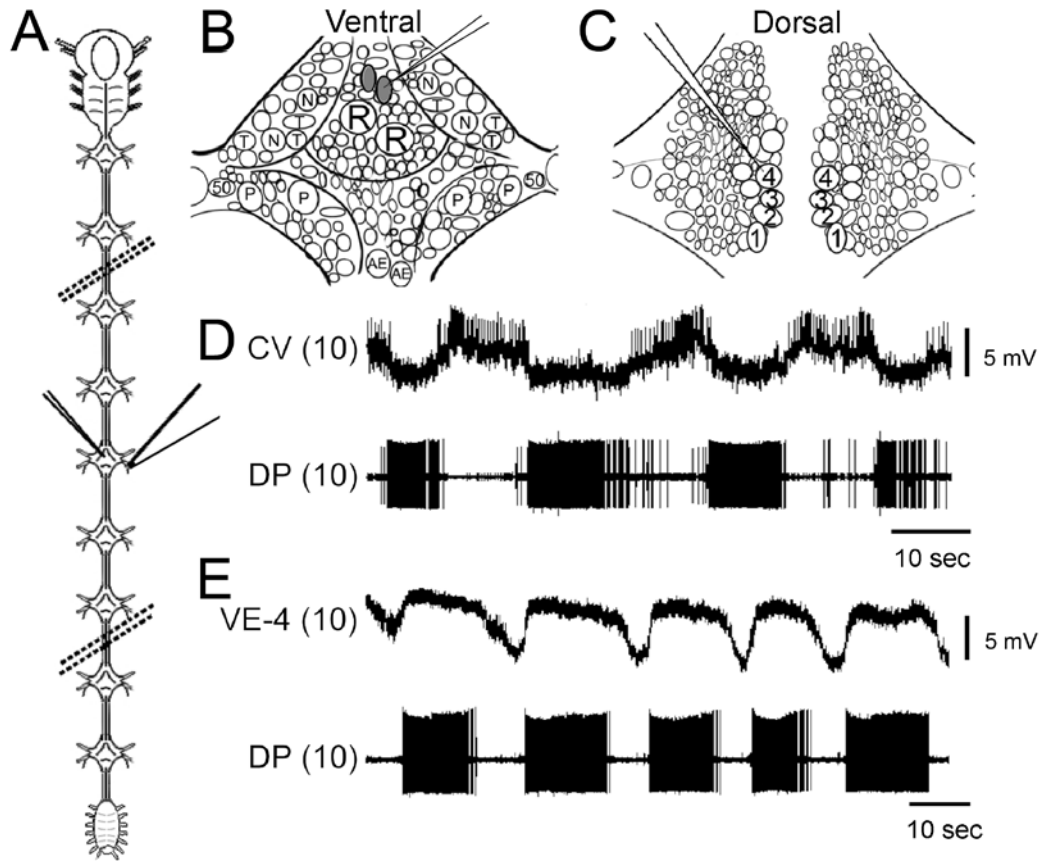


Figure 2-2: Activities of select motoneurons during DA-induced fictive crawling in the whole CNS.

Figure 2-3. Activities of key excitatory motoneurons during DA-induced fictive crawling in single, isolated ganglia. **A-C**: top traces are intracellular recordings from motoneurons and bottom traces are extracellular recordings of the DP nerve, which contains the axon of the dorsal longitudinal excitor motoneuron (DE-3); the largest spike in the DP nerve. Numbers in parentheses denote the segment number. **A**: Intra- and extracellular recording of DE-3, a crawl monitor cell. Lower two traces (shaded in gray) show an expanded time scale and a 1:1 correlation of spikes. DA concentration was 75 μ M. **B**: Ventral longitudinal excitor motoneuron (VE-4). DA concentration was 75 μ M. **C**: Ventrolateral circular excitor motoneuron (CV). DA concentration was 100 μ M.

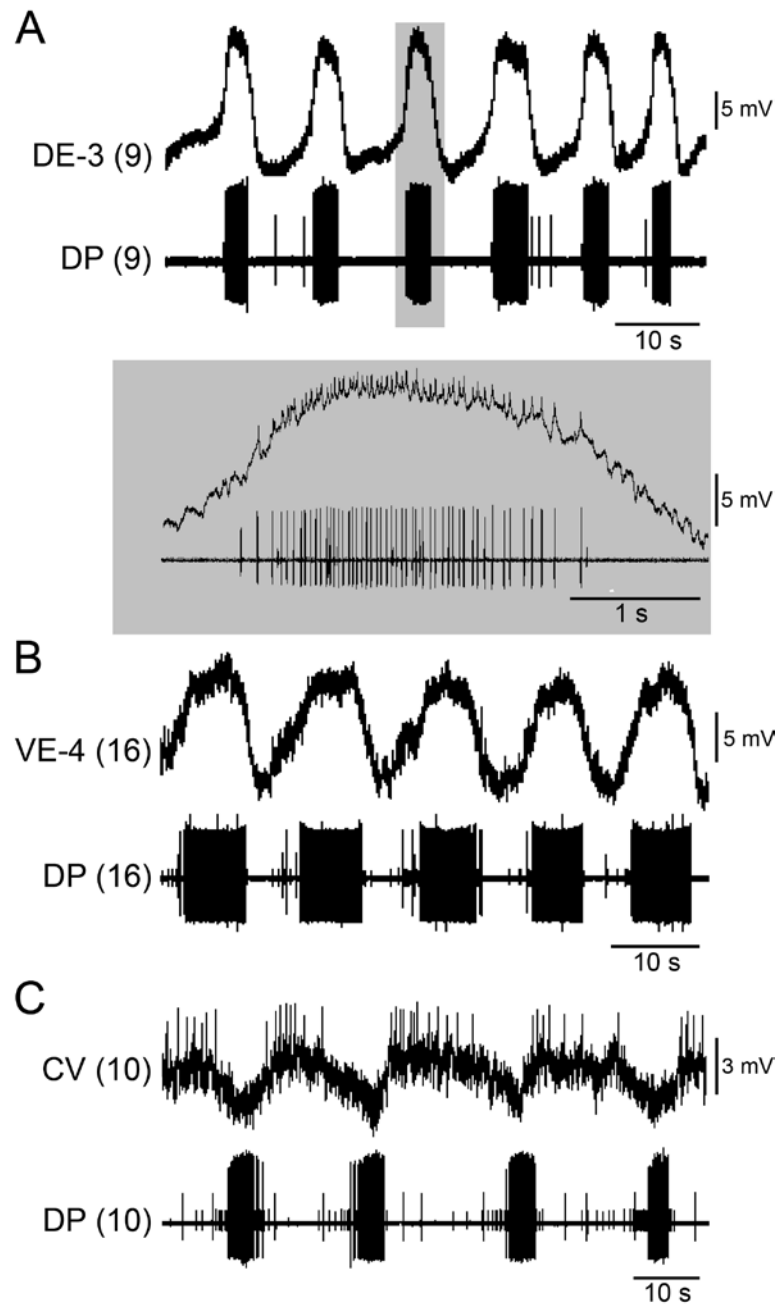


Figure 2-3: Activities of key excitatory motoneurons during DA-induced fictive crawling in single ganglia.

Figure 2-4. Activities of inhibitory longitudinal motoneurons during DA-induced fictive crawling in single, isolated ganglia. **A,B**: top traces are intracellular recordings from motoneurons and bottom traces are extracellular recordings from the DP nerve. Numbers in parentheses denote the segment number. **A**: Dorsal longitudinal inhibitory motoneuron (DI-1). **B**: Ventral longitudinal inhibitory motoneuron (VI-2). For (**A**) and (**B**), the DA concentration was 75 μ M.

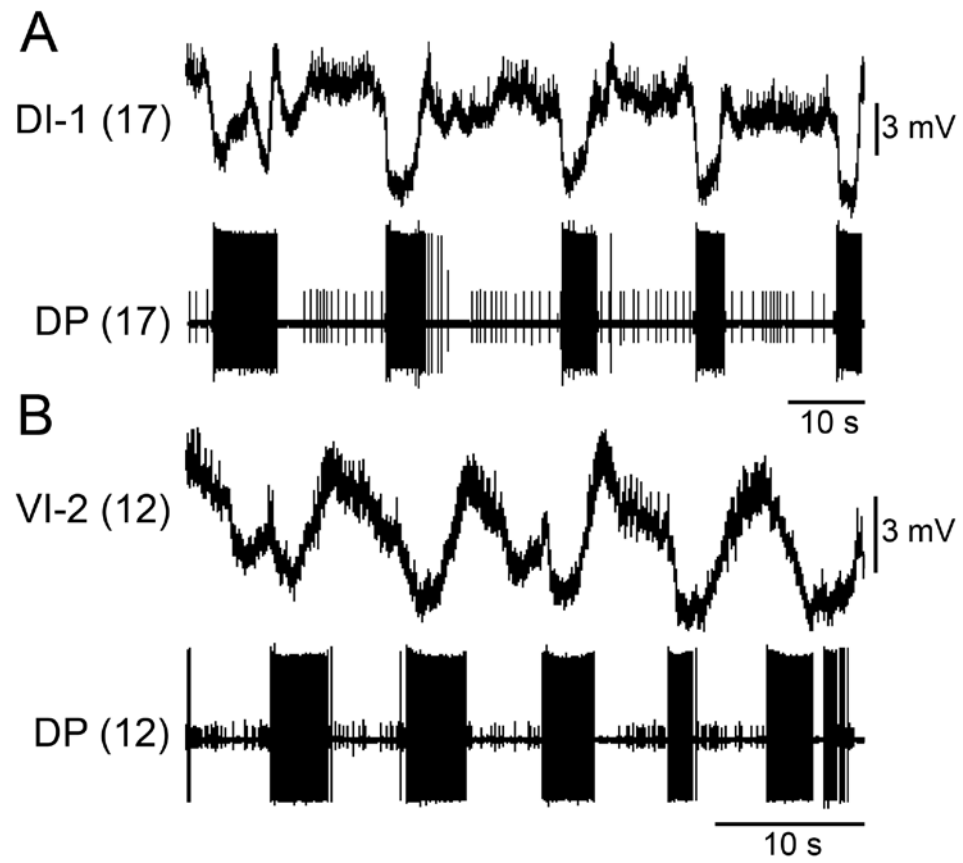


Figure 2-4: Activities of select inhibitory motoneurons during DA-induced fictive crawling in single ganglia.

Figure 2-5. Crawl-like bursting activity of motoneuron DE-3 recorded in the DP nerve of a single isolated ganglion obtained from either an anterior (**A**) or posterior (**B**) body region. Each ganglion was superfused with 100 μ M DA. **A**: Extracellular recording of the DP nerve root from ganglion M2. **B**: Extracellular recording of a DP nerve root from ganglion M19. Numbers in parentheses denote the segment number.



Figure 2-5: Crawl-like bursting activity of single ganglia obtained from anterior or posterior body regions.

Figure 2-6. Features of DA-induced fictive crawling in the single, isolated ganglia. **A:** Percentage of single, isolated ganglia that produced fictive crawling in response to varying DA concentrations (log scale). DA concentrations of 75-100 μ M were determined to be optimal. **B:** Latency to onset of DA-induced fictive crawling in single, isolated ganglia (n = 20) superfused with 75-100 μ M DA. **C:** Duration of uninterrupted bouts of DA-induced fictive crawling in single, isolated ganglia (n = 20) superfused with 75-100 μ M DA.

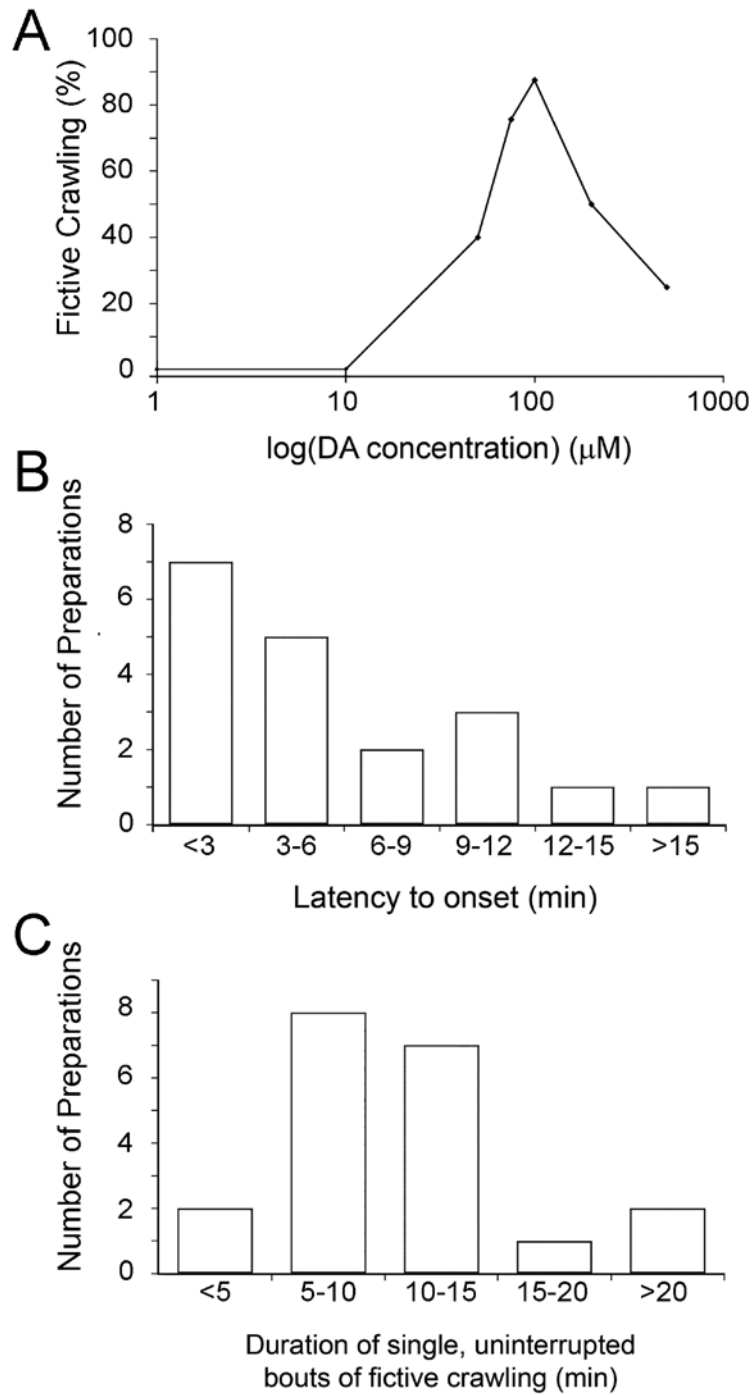


Figure 2-6: Features of DA-induced fictive crawling in single ganglia

Figure 2-7. Comparisons of parameters of the crawl rhythm in: untreated whole nerve cords (solid black bars), whole nerve cords treated with DA (striped bars), DA-treated single ganglion (cross-hatch bars), and intact animals (solid grey bars). The sample size for all groups was 10 animals. **A:** Comparison of crawl periods. No statistically significant differences were found between fictive crawl groups (left 3 bars; $p = 0.14$). A significant difference was found between overt crawling (right bar) and fictive crawling periods ($p < 0.001$). **B:** Comparison of DE-3 burst durations; no significant differences among the groups were found ($p > 0.5$). **C:** Comparison of DE-3 (contraction) duty cycles. No significant differences among the groups were found ($p > 0.1$).

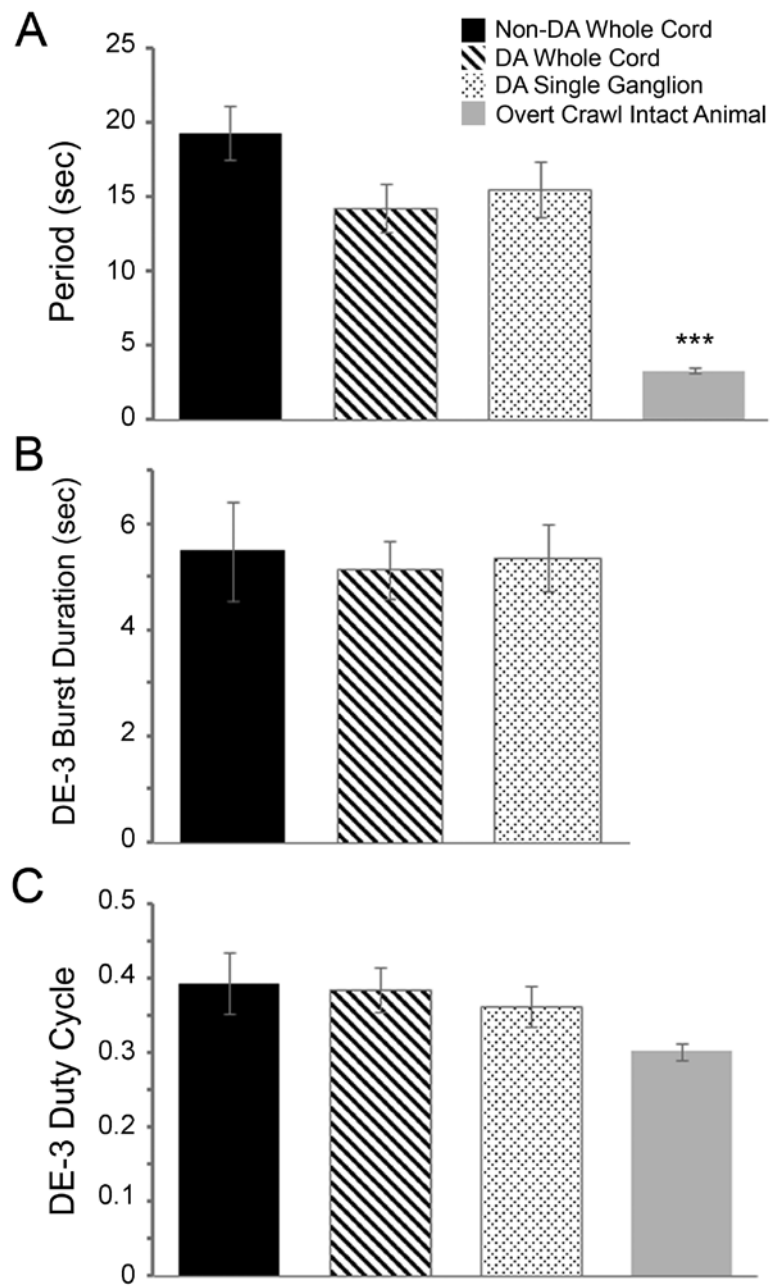


Figure 2-7: Comparisons of parameters of the crawl rhythm.

Figure 2-8. Period dependence of DE-3 burst duration and overt crawl contraction duration. Data points are averaged values from a single animal. Linear regressions were performed using the least squared method (R^2 values are shown in the upper left corner of each panel) and the resulting regression lines are shown in each panel. **A-C:** DE-3 burst duration vs. the period for the three fictive crawling groups. **A:** Spontaneous or electrically-induced fictive crawling in the whole CNS ($n = 10$). The slope of the regression line was 0.24 and the y-intercept was 0.90. **B:** DA-induced fictive crawling in the whole CNS ($n = 10$). The slope of the regression line was 0.19 and the y-intercept was 2.48. There was a weak relationship between the DE-3 burst duration and the period for fictive crawling in (**B**). **C:** DA-induced fictive crawling in the single, isolated ganglion ($n = 10$). The slope of the regression line was 0.45 and the y-intercept was -1.30. **D:** Contraction duration vs. period for overt crawling in intact leeches ($n = 10$). The slope of the regression line was 0.28 and the y-intercept was 0.04. In (**C**) and (**D**), a stronger linear relationship existed between the period and the DE-3 burst duration (DA-treated single ganglion) and the contraction duration (intact animal).

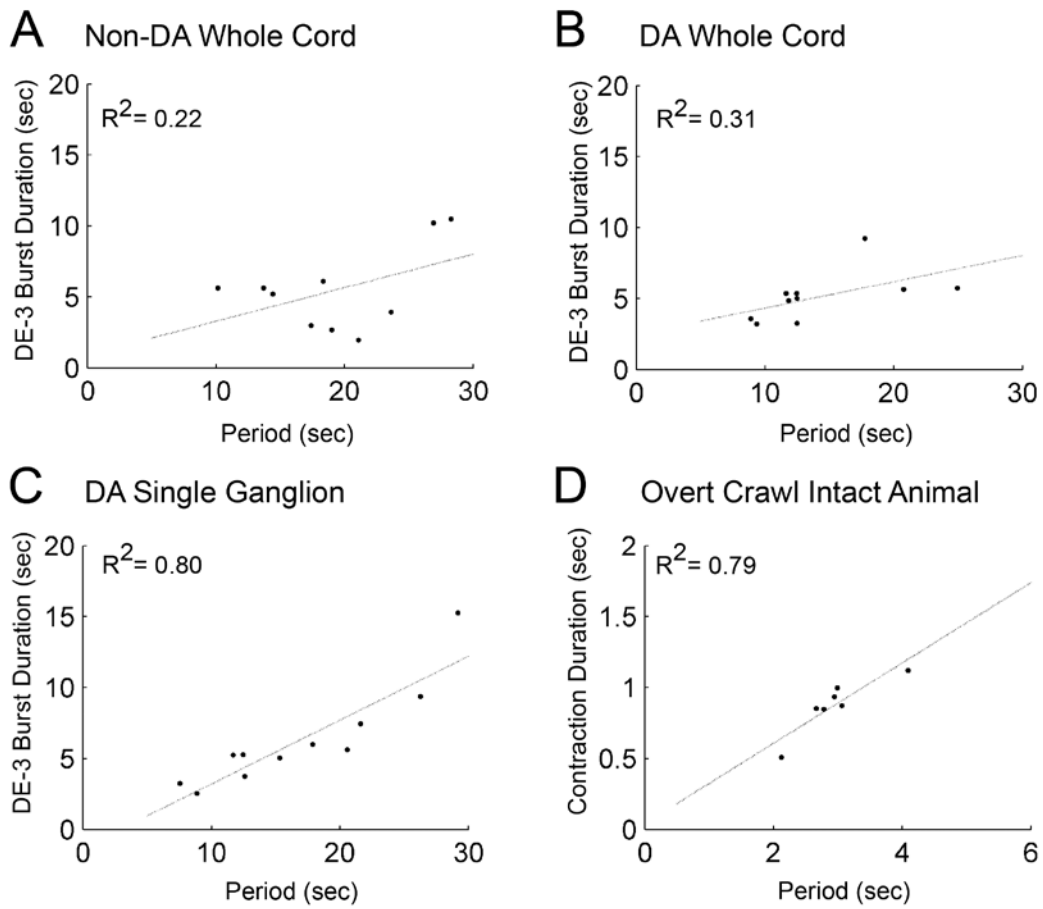


Figure 2-8: Period dependence of DE-3 burst duration and overt crawl contraction duration.

Chapter Three:

Keeping it together: Mechanisms of intersegmental coordination for a flexible locomotor behavior

Puhl JG, Mesce KA (2010) Keeping it together: Mechanisms of intersegmental coordination for a flexible locomotor behavior. J Neurosci 30:2373-2383.

The coordination of multiple neural oscillators is key for the generation of productive locomotor movements. In the medicinal leech, we determined that activation and coordination of the segmental crawl oscillators, or unit burst generators, are dependent on signals descending from the cephalic ganglion. In nearly-intact animals, removing descending input (reversibly with a sucrose block) prevented overt crawling, but not swimming. Cephalic depolarization was sufficient for coordination. To determine whether descending signals were necessary for the generation and maintenance of posterior-directed intersegmental phase delays, we induced fictive crawling in isolated whole nerve cords using dopamine (DA) and blocked descending inputs. After blockade, we observed a significant loss of intersegmental coordination. Appropriate phase delays were also absent in DA-treated chains of ganglia. In chains, when one ganglion was removed from its neighbors, crawling in that ganglion emerged robust and stable, underscoring that these oscillators operate best with either all or none of their intersegmental inputs. To study local oscillator coupling, we induced fictive crawling (with DA) in a single oscillator within a chain. Although appropriate intersegmental phase delays were always absent, when one ganglion was treated with DA, neighboring ganglia began to show crawl-like bursting, with motoneuron spikes/burst greatest in untreated posterior ganglia. We further determined that this local excitatory drive excluded the swim-gating cell, 204. In conclusion, both long-distance descending and local inter-oscillator coupling contribute to crawling. This dual contribution helps to explain the inherent flexibility of crawling, and provides a foundation for understanding other dynamic locomotor behaviors across animal groups.

Introduction

Animal locomotion occurs in many forms and encompasses a wide variety of rhythmic movements. Yet, across this array of activities, it is the coordination of multiple neural oscillators that is needed to generate fluid and productive locomotor behavior (Grillner, 2003). A core feature of this coordination is the so called 'coupling' of central pattern generators (CPGs) within the central nervous system (CNS), which can employ one or more key strategies: 1) Coupling that is inherent to the rhythm-generating circuitry; leading to greater stereotypy in the phase relationships between oscillators, and 2) Coupling that is extrinsic to the unit oscillators. This latter strategy is often associated with a greater flexibility in behavioral output, but at the expense of the predictability provided by the first one. Because stereotypy is a beneficial attribute that renders a behavior more amenable to a physiological analysis, many studies of locomotion have revealed neural operations that are comprised of relatively tightly coupled oscillators. For example, experimental and modeling studies of swimming in the lamprey and leech, insect flight and swimmeret beating in the crayfish have provided much of the foundation underlying what is currently understood about the coordination of oscillators whose coupling is an emergent property of the oscillators themselves (Ikeda and Wiersma, 1964; Cohen and Wallen, 1980; Robertson and Pearson, 1983; Cang and Friesen, 2002; Smarandache et al., 2009). In contrast, other locomotor behaviors exhibiting much more variability are not as well understood at the cellular level (Kiehn and Butt, 2003).

Studies of walking in insects (Büschges et al., 2008), cats (Büschges, 2005) and humans (Yang and Gorassini, 2006), and those of crawling in the medicinal leech (Cacciatore et al, 2000), suggest that these locomotor events may employ oscillator-coupling strategies to maximize flexibility. Some research has indicated that higher-

order descending information may contribute to oscillator coupling for both leech crawling (Cacciatore et al., 2000; Cornford et al., 2006) and cat walking (Shimamura and Kogure, 1983; Drew et al., 1986), although more direct evidence is warranted. In the cat and human, investigations of oscillator coupling and descending control have been limited by the inherent complexities of the mammalian CNS and the locomotor behaviors themselves. Thus, to understand better how a relatively slow and variable locomotor behavior is controlled, we turned to a study of crawling behavior in the medicinal leech.

Briefly, leech crawling consists of alternating elongation and contractions of the whole body with coordinated attachment and release of the anterior and posterior suckers. Segmental movements always progress along the body in an anterior to posterior direction, but they are often dynamic and can be suspended or modulated during each elongation cycle, often with other behaviors overlaid (e.g., searching-related hyperextensions, lateral turning, and dorsal-ventral undulations). Importantly, after a pause in the elongation phase, the movement is often completed from the start of the initial pause (Cacciatore et al., 2000). This flexibility resembles that observed during walking of the stick insect (Gruhn et al., 2009), cat (Akay et al., 2006) and human (Ivanenko et al., 2009). Recently, we established that every segmental ganglion of the leech ventral nerve cord contains a complete crawl oscillator (CPG), which can be independently activated (Puhl and Mesce, 2008). Because of this anatomically favorable situation, we examined the nature of the coupling of these oscillators and determined the role of higher-order descending information to choreograph their activation and coordination.

Materials and Methods

Animals and physiological solutions

Adult medicinal leeches (*Hirudo verbana*) (Siddall et al., 2007) weighing between 1.5-3.0 g were obtained from either Leeches USA (Westbury, NY, USA) or Niagara Medical Leeches (Niagara Falls, NY, USA). Leeches were maintained, at room temperature, in distilled water containing dilute Instant Ocean sea salts (1.89 g/gallon; Spectrum Brands, Atlanta, GA).

Leeches were anesthetized in ice-chilled saline prior to dissection. All dissections and electrophysiological recordings were performed at room temperature in chilled normal saline. Normal saline contained (in mM/L) 116.0 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.5 MgCl₂, 10.0 dextrose, 10.0 Trizma preset crystals, pH 7.4 (Sigma-Aldrich, St. Louis, MO) (adapted from Nicholls and Baylor, 1968). To block chemical synaptic transmission a 20:1 Mg⁺⁺/Ca⁺⁺ ratio saline was used (mmol/L): 87.0 NaCl; 4.0 KCl; 1 CaCl₂; 20 MgCl₂; 10.0 dextrose; Trizma preset crystals, pH 7.4 (Szczupak et al., 1998). Saline containing dopamine (DA) was made fresh daily and used within 5 hours. To obtain final DA concentrations of 50-100 μM, a 1 mM DA stock was diluted with normal saline immediately prior to use (Puhl and Mesce, 2008).

Overview of leech neuroanatomy and nomenclature

The nervous system of the leech consists of a compound cephalic ganglion (i.e., the supraesophageal and subesophageal ganglia), 21 segmental ganglia (designated M1-M21), and a compound tail ganglion (Fig. 3-4A). Connectives joining these ganglia are comprised of two hemi-connectives and a smaller median Faivre's nerve (FN).

Each segmental ganglion contains a dorsal and ventral layer of neuronal somata. Key somata of uniquely identifiable motoneurons (MNs) active during crawling are: the

ventral lateral circular excitor (CV) MNs and the dorsal longitudinal excitor (DE-3) MNs, located on the ventral and dorsal surfaces respectively (Stuart, 1970).

Experimental preparations and nomenclature

In this study, locomotor behaviors were monitored in both nearly-intact and isolated CNS preparations. The nearly-intact preparation allowed for observation of overt locomotion while providing direct access to the CNS (Esch et al., 2002). The term “isolated whole nerve cord” refers to a complete CNS preparation dissected from the body, while the term “chain preparation” refers to a subset of isolated ganglia lacking the cephalic and tail ganglia. To observe fictive locomotion in isolated or chain CNS preparations, motor patterns were monitored by recording the extracellular activity of DE-3, whose large axon resides in the dorsal posterior (DP) nerve roots of each ganglion. The DE-3 spike is typically the largest unit in the DP recordings (Kristan et al., 1974). We defined fictive crawling as three or more consecutive bursts of DE-3 with periods ranging from 5-25 s (Eisenhart et al., 2000; Puhl and Mesce, 2008).

For isolated whole nerve cord preparations, the entire intact CNS was removed with both left and right DP nerves from 2-4 segmental ganglia. Shorter chain preparations were made by removing 4-6 segmental ganglia, ensuring the connectives remained intact. These chains were taken from M7-M18 including both left and right DP nerves from at least 2-3 segmental ganglia. When necessary, a Vaseline well was placed around individual ganglia, isolating them for focal DA application. In some experiments, the FN was manually severed or crushed using a fine forceps, leaving both hemi-connectives intact. The hemi-connectives were severed by cutting them with fine-tipped scissors, leaving only the FN intact.

Briefly, for the nearly-intact preparation, the body tissues surrounding the cephalic ganglion through ganglion M3 were removed while keeping the remaining body intact. The nerve roots of the tail ganglion and M4 were severed. The preparation was stabilized in a two-chambered dish by anchoring the anterior edge of the body wall to a wax pad with the intact portion of the animal placed in the larger posterior chamber, leaving it free to locomote (see Fig. 3-2A, schematic). The fluid levels of the posterior chamber could be changed from shallow (< 0.1 cm) to deep (1.0 cm). We observed spontaneous crawling, as it was produced by the nearly-intact leech, or we elicited crawling by gently pulling on the rear of the leech with a small applicator stick to elongate its body, which was shown previously to produce crawling reliably (Esch et al., 2002). The applicator stick was gently applied to the dorsal aspect of the body to elicit swimming.

Sucrose block, ion exchange block and focal K⁺-induced excitation

We employed the sucrose block technique of Masino and Calabrese (2002) to reversibly block signals descending from the cephalic ganglion in nearly-intact and isolated whole nerve cords. The sucrose delivery system was modified by constructing a bowtie-shaped well made of Sylgard (Dow Corning, Midland, MI), which was placed across the connectives between two ganglia (M2-M3). Neural transmission was blocked when the well's saline was replaced with an isotonic sucrose solution (260 mM) that lacked the ions necessary for neural transmission (e.g., Na⁺, K⁺, Ca²⁺). In our hands, we confirmed the effectiveness of the sucrose block by demonstrating that neural transmission was eliminated in sucrose and restored when the well was replenished with normal saline (Fig. 3-1).

To block chemical synaptic activity within a single ganglion while not disrupting fibers-of-passage, a given ganglion was isolated within a Vaseline well and bathed for 15-20 min in a saline solution containing a high Mg^{++} -low Ca^{++} (recipe provided above). Similar methods have been used in the leech and crayfish to silence individual segmental CPGs for locomotion (Pearce and Friesen, 1985; Tschuluun et al., 2001). To increase the general excitation of the cephalic ganglion, a 2M K^+ acetate/20 mM K^+ chloride solution was briefly applied; a similar method was used by Friesen and Brodfuehrer (1984) to depolarize cephalic leech neurons. The solution was washed away within several seconds of application by flow of the saline-containing superfusion system.

Electrophysiological recordings and cell identification.

Extracellular unit activity was recorded from a chosen DP nerve using suction electrodes (~ 50 μm tip width). Extracellular signals were amplified using a Grass Technologies P15 AC preamplifier (West Warwick, RI) and recordings were digitized (10,000 samples/s) using a MacLab/4s data acquisition system (ADInstruments, Bella Vista, New South Wales, Australia) attached to a Microsoft Windows-compatible personal computer. Activity of DE-3 in all DP recordings served as a monitor of fictive crawling.

The intracellular activities of identified MNs were recorded using a Dagan (Minneapolis, MN) IX2-700 amplifier and digitized as described above. Electrodes were filled with a solution of 2 M potassium acetate and 20 mM potassium chloride and had resistances of 40-65 M Ω . For cell labeling, intracellular pipette tips were back filled with a 2 M potassium acetate solution that contained 5% (w/v) Neurobiotin (Vector Laboratories, Burlingame, CA).

MNs were identified by their electrophysiological signatures and the position and size of their somata. Cell identities were confirmed by iontophoretically injecting 5% Neurobiotin (0.5-1 nA) into a given cell for 10-30 min. Tissues were fixed, processed, treated with 1:50 Cy3-conjugated Streptavidin (Jackson ImmunoResearch, West Grove, PA) in PBS for 24 h at 4°C and prepared for viewing according to the methods of Gilchrist et al. (1995).

Data analysis and statistical methods

To determine the temporal properties of fictive crawling, we analyzed bursting activity of DE-3. A grouping of DE-3 spikes was deemed a burst if a minimum of 10 spikes had less than 2 s between them, although most bursts contained dozens of spikes with more than 5 s of separation. Crawl periods were calculated by measuring onsets of sequential DE-3 bursts. If burst start times were not clearly discernable (< 15% of samples), burst midpoints were used to calculate the period. To determine midpoints, DE-3 spike frequency over time was calculated by counting the number of DE-3 spikes per 250 ms time bin and multiplying by 4 bins/s (spikes/s). For each preparation, a spike frequency threshold was calculated by adding one standard deviation of the DE-3 spike frequency to the minimum spike frequency observed during the analysis window. Burst start and end times were when the spike frequency crossed threshold. The midpoint of each burst was calculated by adding 0.5 times the burst duration to the burst start times. Mean crawl periods were determined by analyzing 6-10 consecutive crawl cycles. Intersegmental delays (ISDs) were determined by subtracting posterior DE-3 onsets from anterior ones and dividing by the number of segments separating the two. Mean ISDs were determined by analyzing 5-13 consecutive crawl cycles. Burst midpoints were used to calculate ISDs *in lieu* of burst start times if burst

onsets were not clearly discernable. Intersegmental phase delays, in degrees per segment, were calculated by multiplying the ISD by 360° and dividing by the corresponding crawl period. For ISD and phase delay calculations, the most anterior DP recordings were used as the reference signal.

The Mann–Whitney–Wilcoxon (MWW) nonparametric test was used to assess whether two independent samples came from the same distribution and was performed using the free software package R (R Development Core Team, 2009). All statistical tests were two-tailed with a 95% confidence level ($\alpha = 0.05$). Sample means were presented \pm the standard error of the mean (SEM).

Results

Descending signals from the cephalic ganglion are necessary for overt crawling in nearly-intact leeches

Figure 3-1 demonstrates the effectiveness and reversibility of the sucrose block. By placing this block below the cephalic ganglion, we determined the necessity of cephalic signals for overt crawling behavior in nearly-intact leech preparations (Fig. 3-2A,B). Figure 3-2B depicts the contraction and elongation phase of one overt crawl cycle. Spontaneous crawling behaviors were monitored when the body of the leech was situated in either shallow or deep water, and measured prior to the sucrose blockade (Pre-Block), during the block (Blocked) and after the sucrose was exchanged with normal saline (Post-Block). We observed no spontaneous overt crawling during the Block in either shallow (black bars) or deep water (gray bars) (Fig. 3-2C). In shallow water the number of spontaneous crawl cycles was significantly less compared to the Pre-Block and Post-Block conditions ($p < 0.01$) (Fig. 3-2C). Spontaneous crawling in deep water was limited and only observed during the Pre-Block phase (Fig. 3-2C).

Although these data indicate that descending cephalic signals are necessary for spontaneous overt crawling, they did not establish whether their absence rendered leeches incapable of executing crawling when stimulated. To study this possibility, we attempted to elicit crawling by gently hyperelongating the posterior body with a small applicator stick (Esch et al., 2002). During the Pre-Block phase, in shallow water, crawling was elicited in all animals ($n = 8$) in 100% of trials (5 out of 5 attempts) (Fig. 3-2D). During the Blocked phase, it was not possible to elicit a single cycle of crawling in any of the animals tested (Fig. 3-2D). During this phase, we attempted to elicit crawling by pricking the cuticle with a metal pin just anterior to the tail sucker ($n = 3$). Under this noxious stimulation, 100% of preparations failed to crawl, although the stereotypic shortening response was exhibited (Kristan et al., 1982; data not shown). During the subsequent Post-Block phase, in shallow water, crawling was stimulated in 5 out of 5 attempts among all animals tested (Fig. 3-2D). These data provide clear evidence that descending signals from the cephalic ganglion are necessary for the expression of overt crawling in nearly-intact animals.

Crawling and swimming are differentially dependent on descending signals from the cephalic ganglion.

In this companion study, we determined if the removal of descending signals also prevented swimming. Although a number of previous reports had shown that swimming can occur in the absence of the cephalic ganglion, they involved surgical manipulations that were irreversible (Brodfuehrer and Friesen, 1986a; Cornford et al., 2006). Thus, we analyzed spontaneous and evoked overt swimming behavior using the reversible sucrose block. We conducted our analysis in shallow and deep water using the same

animals analyzed in the previous section. Because no leeches swam in shallow water, data only for the deep water condition were plotted (Fig. 3-3).

Spontaneous swimming was observed in all animals tested in all three conditions (Fig. 3-3A-C; $n = 8$). The number of swim episodes (bouts) observed during the blockade of cephalic signals was significantly different from the Pre- and Post-Block phases ($p < 0.01$) (Fig. 3-3A), although no significant differences in the total number of swim cycles was found between the three phases ($p > 0.5$; Fig. 3-3B). It is noteworthy that the number of swim cycles within an individual swim episode was significantly larger ($p < 0.05$) during the Blocked phase (127.9 ± 57 cycles per episode) as compared to the Pre- and Post-Block phases (Fig. 3-3C; 13.9 ± 3.8 and 24 ± 5.9 cycles per episode, respectively).

To examine evoked swimming, we gently stroked the dorsal surface of the leech's body. In nearly all attempts (out of 5), swimming was elicited during the Pre-Block, Blocked and Post-Block phases (Fig. 3-3D). These data demonstrate that crawling and swimming are differentially dependent on descending cephalic signals, and are consistent with a previous report indicating that the cephalic ganglion may play a predominantly suppressive role in controlling swimming behavior (Brodfuehrer and Friesen, 1986a).

Cephalic descending signals are necessary and sufficient for coordinated fictive crawling biased by dopamine

Based on the results of the previous section we hypothesized that descending inputs provide multiple levels of regulation: 1) a system-wide activation and maintenance of the independent segmental crawl oscillators and 2) the setting of the intersegmental phase relationships during each crawl cycle. To understand the specific contributions of

descending inputs for oscillator coordination, we needed first to bias the CNS in the direction of crawling while simultaneously suppressing swimming so that these questions could be addressed. This strategy was best achieved by applying 50-100 μM DA (Crisp and Mesce, 2004; Puhl and Mesce, 2008) to isolated whole nerve cords and monitoring fictive crawling via 2 or more DP nerves separated by a given number of segments (Fig. 3-4A). We again used the sucrose block to reversibly remove cephalic descending signals; in addition, in some preparations we focally stimulated the cephalic ganglion to initiate fictive crawling.

In Figure 3-4B, we show activity of the crawl-monitor cell, DE-3, across 5 ganglia (DP nerves 10, 12 and 14) in the absence (Block ON) and then presence (Block OFF) of cephalic descending inputs. The isolated whole nerve cord was biased to crawl by applying 50 μM DA, a concentration at the threshold for inducing crawling (Puhl and Mesce, 2008). At the beginning of the recordings shown (Fig 3-4B, far left arrow), the cephalic ganglion was depolarized with K^+ while the sucrose block was in effect; note the lack of any changes in recorded activity. When the block was subsequently removed (Fig 3-4B, Block OFF), robust and coordinated fictive crawling emerged across all three segments. In a complementary experiment, we stimulated the cephalic ganglion focally with K^+ (Fig. 3-4C, arrow) to demonstrate that cephalic activation is sufficient to induce coordinated fictive crawling when descending inputs are present (note: DE-3 bursts showed no intersegmental coordination prior to cephalic stimulation). This stable and robust metachronal crawling was subsequently abolished in the presence of the block (Fig. 3-4D).

Analysis of crawl disruption when cephalic inputs were removed

In isolated whole nerve cord preparations receiving the sucrose block, DA-induced fictive crawl-like bursts were either intermittent or ceased altogether [i.e., stopped crawling (SC) ($n = 5$)] or exhibited altered intersegmental coordination ($n = 13$) (Fig. 3-5A). The removal of cephalic inputs resulted in a spectrum of changes whereby ISDs changed but cycle periods remained fairly constant (Fig. 3-5B, $n = 9/13$) or periods changed within and between segments (Fig. 3-5C, $n = 4/13$), all of which abolished the metachronal intersegmental phase relationships. During experiments in which recorded crawl bursts could readily be discerned across all recorded segments (e.g., Fig. 3-5B), intersegmental phase delays were calculated and differed significantly in Pre-Blocked and Blocked conditions (Fig. 3-5D). Statistical significance between intersegmental phase delays of Pre-Block and Blocked conditions was determined for each preparation. In preparations that showed more pronounced alterations (Fig. 3-5C), changes in intersegmental coordination were assessed by measuring the period of DE-3 crawl bursts within and across segments. Figure 3-5E shows changes in the intra- and intersegmental periods for 3 different preparations (*i.-iii.*) prior to and during the sucrose block. During Pre-Block conditions, all 3 nerve cords revealed isochronicity (i.e., a fairly constant period), and all crawl oscillators within a preparation exhibited a similar crawl period (Fig. 3-5E, note flat and overlapping lines). Figure 3-5E (*i*) illustrates that even when one crawl period transiently deviated from its set point (in Pre-Block), all 3 segmental crawl oscillators shared the same deviation. Upon removal of descending signals, however, the stability of these periods was lost within and across the segmental oscillators (Fig. 3-5E, Blocked). This block-induced variability in crawl period precludes the formation of appropriate phase delays and intersegmental coordination. In addition, we calculated the correlation of ISDs and period length for 80 crawl cycles ($n = 8$

preparations) in control leeches and found a coefficient of correlation to be 0.73. This result indicated that the ISD increases with cycle period. For overt crawling, Stern-Tomlinson et al. (1986) reported that ISDs also increase as crawl periods lengthen. Even if one were to argue that the loss of cephalic inputs translates into a decline in excitatory drive and slower crawl period, theoretically ISDs should still be able to adjust to changes in crawl period, but as shown in Figure 3-5C the ISDs differ over successive cycles, indicating a disruption in coordination.

Finally, we tested whether removal of ascending information from the compound tail ganglion affects the intersegmental coordination of crawling. We used the sucrose block to suppress signals from the tail ganglion. We induced sustained fictive crawling by treating whole nerve cords with 75-100 μ M DA and observed that blockade of ascending signals had no significant effects on the ISDs and periods of fictive crawling (data not shown; $n = 4$).

Crawl oscillators within chains of ganglia: lack of intersegmental coordination and stability

In our previous study (Puhl and Mesce, 2008), we demonstrated that a single segmental ganglion can express robust and stable fictive crawling when treated with DA. Crawl periods within and across ganglia were similar (Puhl and Mesce, 2008). Based on this information, one might predict that a DA-treated chain of only a few ganglia would reveal more stable crawling; furthermore, as isolated chains they would provide yet another test of the need for cephalic descending signals to coordinate the iterated oscillators.

We observed that a DA-treated chain of 5 ganglia, lacking the cephalic ganglion, never exhibited coordinated crawling with appropriate intersegmental phase delays (Fig.

3-6A) ($n = 5$). Although each segmental crawl oscillator gave rise to DE-3 bursting in each segment (Fig. 3-6A), the intra- and intersegmental timing events never mirrored those in the isolated ganglion or whole nerve cord. Thus, the crawl oscillators appear to be most stable either in complete isolation or in the presence of intact descending inputs. One hypothesis is that any intermediate variants in input perturb the crawl system as a whole. An alternative hypothesis is that the crawl oscillators in chains lack sufficient descending cephalic drive needed to activate DA-biased crawling. To test these ideas further, we removed the end ganglion (in the chain shown in Figure 3-6A) so that it was freed from all intersegmental inputs (Fig 3-6B, left diagram). As predicted by our first hypothesis, we found that a strong crawl rhythm emerged (Fig. 3-6B); it became even more robust and stable over time in isolation (Fig. 3-6C).

Role of individual oscillators for intersegmental crawl drive

At this juncture, it is clear that the cephalic ganglion plays a vital role in coordinating the segmental crawl oscillators, thus generating the posterior-directed metachronal waves of motor activity during each crawl cycle. Furthermore, DA-induced crawling is most unstable when only a sub-set of intersegmental signals is present, but Figure 3-6A also hints at the idea that the crawl oscillators influence the cycling frequency of nearby crawl oscillators. To begin to identify shorter-distance intersegmental signals, we asked whether individual crawl oscillators are capable of influencing or driving crawl-like activity in neighboring ganglia.

To address this question, we recorded DP nerve activity in chains of 4-6 ganglia from either the middle (M7-M14) or posterior (M15-M20) body regions, and exploited the ability of DA to activate a single crawl oscillator; we then measured the influence of one oscillator on its segmental neighbors. Figure 3-7A shows an experiment in which a

single ganglion (M13, shaded in gray) was induced by DA to express fictive crawling. The DP nerves of three ganglia were monitored simultaneously: the DA-treated ganglion (denoted G/DA) and the anterior (G/-1) and posterior (G/+1) adjacent ganglia (Fig 3-7A, diagram at left). Prior to DA treatment, rhythmic DE-3 bursting was absent in all three DP recordings (data not shown). Shortly after DA was applied normal fictive crawling developed in the treated ganglion with a period of approximately 22 s (Fig. 3-7A, shaded middle trace). In G/-1 (not treated with DA), we observed distinct DE-3 bursts with regular cycle periods of approximately 23 s, resembling fictive crawling (Fig. 3-7A, top trace). The intra-burst firing rate of DE-3, however, was lower than what would typically be expected in ganglia induced to crawl with DA (Puhl and Mesce, 2008). In G/+1, DE-3 was much more active, exhibiting a rhythmic modulation of its spiking frequency with a period of ca. 22 s (Fig. 3-7A, bottom trace), but individual pseudo-bursts were not distinct. It is noteworthy that we did not observe normal intersegmental phase delays of DE-3 bursting in any of the DA-treated chain preparations we examined ($n = 60$). In some preparations, however, the burst frequencies of DA-treated and non-treated ganglia were similar.

Although the DE-3 bursting in G/-1 and G/+1 resembled fictive crawling, several parameters needed to be fulfilled to substantiate this idea quantitatively. One key criterion to fulfill was that the circular (CV; elongation phase) and longitudinal (DE-3; contraction phase) MNs burst $\sim 180^\circ$ out of phase, as they do during DA-induced fictive (Puhl and Mesce, 2008) and overt crawling (Gray et al., 1938; Stern-Tomlinson et al., 1986). Figure 3-7B shows a representative example of DE-3 (bottom trace) and CV (intracellular, middle trace) in G/+1, showing that the spiking activity of CV decreased and the cell was hyperpolarized when DE-3 firing was elevated (note: this ganglion was not treated with DA). We also observed a similar anti-phasic relationship of CV and DE-3

in G/-1 (Fig. 3-7C). Note, however, that in G/-1 the CV hyperpolarization is weak (Fig. 3-7C), suggesting that local crawl drive may be promoted less in the anterior direction and more in the posterior one.

Figure 3-7D provides information about the total numbers and percentages of chain preparations in which DE-3 bursts were elicited in DA-treated and neighboring untreated ganglia; as mentioned crawl-like bursts were more reliably observed in posterior directions. Figure 3-7E shows the percentage of preparations showing anti-phasic CV and DE-3 activity, and G/+1 again shows this feature more reliably as compared to G/-1. Furthermore, although the DE-3 crawl burst periods were indistinguishable across all segments and conditions (Fig. 3-7F), the number of DE-3 spikes/burst were significantly elevated in G/+1 and fewer in G/-1 (Fig. 3-7G). All values were within normal range of fictive crawling (Eisenhart et al., 2000; Puhl and Mesce, 2008).

The extent of local intersegmental drive

To determine the potential extent of crawl-like drive to more distant ganglia, we examined DP nerve activity anteriorly in G/-2, and posteriorly in G/+2 and G/+3. Figure 3-8A shows a record of DE-3 crawl-like bursting in G/+2 (bottom trace). Although many of the bursts across the 3 segments appear co-active, they lack appropriate phase delays. The percentage of preparations exhibiting crawl-like DE-3 bursting is shown in Figure 3-8B. No bursts resembling true fictive crawling were observed in G/-2 (Fig. 3-8B).

To test whether the crawl drive from one segmental oscillator to another was based completely on a concatenated series of inputs from one segment to the next, we induced crawling in one ganglion (G/DA) and blocked chemical synaptic activity in the adjacent posterior ganglion with low Ca^{++} -high Mg^{++} saline (blocked G/+1) to determine

if crawl-like activity could still be communicated to G/+2 ($n = 4$). Figure 3-8C shows the control chain of ganglia prior to treatment exhibiting spontaneous (tonic) activity in DE-3 across all 3 adjacent ganglia. In Figure 3-8D, fictive crawling was induced in G/DA as shown (top trace). At this time, all neural activity was abolished in G/+1 (middle trace) in response to the low Ca^{++} -high Mg^{++} solution (Fig 3-8D). Although no neural activity was recorded from the intervening ganglion (G/+1), the DA-induced crawling in G/DA was able to generate crawl-like bursting in G/+2 (bottom trace), indicating that local intersegmental neurons must span a distance of 2 ganglia or more from their source.

Local crawl-like drive is transmitted via the hemi-connectives

As a first step toward identifying potential local crawl coupling and/or gating cells, we transected subcomponents of the intersegmental connectives to identify the anatomical route that carries the crawl-like drive. Three bundles of neuronal fibers comprise the connectives that link the individual segmental ganglia to each other: paired hemi-connectives and a singular (medial) Faivre's nerve (FN). The hemi-connectives carry the vast majority of interganglionic fibers. The FN has been shown previously to carry an important gating cell for swimming, cell 204 (Weeks and Kristan, 1978), which conceivably could be multifunctional and help gate crawling between adjacent ganglia

To determine which of these structures carries the fibers mediating the local crawl-like drive, we severed either the FN (Fig. 3-9A, far left diagram) or the hemi-connectives (Fig. 3-9B, far left diagram) between G/DA and G/+1. A 5-ganglia chain was used for both experiments and fictive crawling in a given ganglion was activated with DA (G/DA in 100 μM). The recordings in Figure 3-9A demonstrate that after severing the FN, crawl-like drive (bottom trace) was not disrupted (4 out of 5

preparations). In contrast, when the hemi-connectives were severed in 5 different preparations, no crawl-like activity was recorded in G/+1 (bottom trace) (e.g., Fig. 3-9B).

Discussion

Higher-order descending information and local signals contribute to the intersegmental coordination of locomotor oscillators

In this study of leech crawling, we demonstrated that the compound cephalic ganglion plays a critical role in both the activation and coordination of the segmental crawl oscillators; furthermore, we demonstrated that the individual locomotor oscillators provide excitatory drive to each other (Fig. 3-10 summary diagram). Although a number of computational models have been proposed to explain how long-distance projections might contribute to locomotion (Cohen and Kiemel, 1993; Cacciatore et al., 2000), to our knowledge, ours is the first empirical study to separate the contributions of higher-order and local drive, and intersegmental coordination.

The combination of descending and local control is likely tied to the flexibility inherent to leech crawling and, in some respects, parallels the adaptability observed for more complex locomotor behaviors like walking (Georgopoulos and Grillner, 1989; Lavoie et al., 1995). The dynamic nature of crawling is also based on oscillator independence (Puhl and Mesce, 2008). For productive locomotion, however, the coordination of these unit oscillators must be constrained so that an anterior-to-posterior wave of intersegmental activity is generated (Eisenhart et al., 2000; Puhl and Mesce, 2008). Because leeches do not crawl backwards nor exhibit intermittent positive and negative intersegmental phase shifts, the neural architecture of the animal must be organized to ensure that posterior-directed movements are executed. Our data indicate that information descending from the cephalic ganglion is necessary for the inter-

oscillator phase delays that propagate in the anterior-to-posterior direction (Fig. 3-5). In addition, cephalic descending inputs are sufficient to organize appropriate intersegmental coordination when the individual crawl oscillators are biased to crawl by DA (Fig. 3-4). This higher-order coordination, likely in combination with local inter-oscillator coupling (Figs. 3-7 and 3-8), acts to stabilize the intersegmental crawl periods and direct the rearward progression of intersegmental time delays during each phase (contraction or elongation) of the crawl cycle.

In other animal systems, studies have suggested that descending information is involved in oscillator coordination during locomotion. In walking cats (Shimamura and Kogure, 1983; Drew et al., 1986) and rodents (Gordon and Whelan, 2008), for example, descending signals from the midbrain and brainstem have been shown to contribute to interlimb coordination. Drew and Rossignol (1984) demonstrated that microstimulation of the medullary reticular formation in the thalamic cat, in addition to activating locomotion (Mori et al., 1978; Garcia-Rill and Skinner, 1987), can lead to phase-dependent changes in leg muscle activity that is incorporated into ongoing walking cycles.

Oscillator-to-oscillator coupling

Locomotor behaviors possessing greater stereotypy often appear less dependent on descending information for oscillator coupling. Although reticulospinal neurons in the lamprey, for example, can drive fictive swimming (McClellan and Grillner, 1984), coordinated swimming can be generated in isolated spinal networks if activated chemically or electrically (Cohen and Wallen, 1980). Such work underscores the importance of local oscillator-to-oscillator coupling.

Figures 3-7 and 3-8 demonstrate that individual crawl oscillators activated by DA can promote crawl-like activity in nearby oscillators. We further determined that crawl-like activity originating in a given ganglion extends to at least two posterior ganglia (Fig. 3-8D). These data suggest that this crawl-like drive is not limited to oscillator-to-oscillator interactions that are concatenated, but must also involve interneuronal signaling spanning multiple segments. These results parallel those in other systems in which well-defined segmental oscillators communicate via intersegmental interneurons projecting through multiple segments (Pearce and Friesen 1985, Tschuluun et al., 2001, Smarandache et al., 2009). Because this drive is stronger in the posterior direction, as evidenced by the increased number of spikes/DE-3 burst [proportional to muscle contraction (Mason and Kristan, 1982)] (Fig. 3-7G), the anterior-to-posterior propagation of crawl activity is facilitated. Importantly, this inter-oscillator signaling is not sufficient to produce normal intersegmental phase delays, thus local coupling provides only a supporting role for coordination, a feature that contrasts with the systems just discussed (Pearce and Friesen 1985, Tschuluun et al., 2001, Smarandache et al., 2009).

Asymmetric oscillator coupling is a critical feature governing the directional propagation of motor activity in many locomotor systems; however, most studies have investigated this coupling in tightly-coupled systems, often lacking the flexibility inherent in leech crawling. Swimming behavior in the lamprey and leech, and swimmeret beating in the crayfish, are representative of more tightly coupled oscillators; arising as an emergent property of the locomotor CPGs (reviewed by: Skinner and Mulloney, 1998; Hill et al., 2003). Locomotor behaviors that require more adaptability, such as crawling or walking (Juvin et al., 2005; Borgmann et al., 2009), may preclude the configuration of oscillators that are tightly coupled and whose coordination is predominantly 'built in' to the pattern-generating elements.

Interaction of local and long- distance signals

One computational model of lamprey swimming (Cohen and Kiemel, 1993) suggests that inter-oscillator coupling can be achieved when stronger, shorter-distance, inter-oscillator signals are combined with long-distance projecting signals. This model can generate predictable inter-oscillator phase delays and without the need for precisely timed information stemming from putative long-distance projecting signals. If the inter-oscillator signals are stronger in the posterior direction, propagation will travel only in that direction, as we see in leech crawling. Our study also substantiated the role of a descending long-distance projecting signal.

Similarly, a model specifically for leech crawling predicted the need for a cephalic signal that projects through the length of the CNS (Cacciatore et al., 2000). Also proposed was the posterior-directed excitatory drive, between segments, as substantiated here (see Figs. 3-7, 3-8, 3-10). However, several important predictions of this model were not confirmed here. For example, Cacciatore et al. (2000) proposed that only a subset of ganglia contain the rhythm-generating elements for crawling. As we have shown here and in our previous report (Puhl and Mesce, 2008), a complete crawl pattern-generating circuit exists within every segmental ganglion.

These DA-biased crawl oscillators were found to be most stable either in complete isolation (Fig. 3-6) or when cephalic descending inputs were present (Fig. 3-4). Dopamine-treated collections of ganglia lacking cephalic inputs exhibited degraded crawl-like activity, supporting the idea that conflicting or inappropriate intersegmental signaling emerges when cephalic influences are absent.

Although we did not specifically identify the cephalic neurons that activate crawling and coordinate the intersegmental phase delays, several candidates could fulfill such functions. Most notable are the R3b cephalic projection neurons. In particular,

cells R3b-1 and R3b-3 can activate or reset the crawl rhythm, and they show rhythmic bursting that is phase-locked with spontaneous overt crawling in nearly-intact leeches (Esch et al., 2002; Mesce et al., 2008). All 3 pairs have been shown to project uninterrupted through the nerve cord. Thus, the long-distance-projecting neurons incorporated into the model of Cohen and Kiemel (1993) do have a biological correlate in the leech, and warrant further study in terms of intersegmental coordination.

Crawling, swimming and their modification

The necessity of cephalic input for crawling sharply contrasts with swimming, the other major form of locomotion in the leech. In the nearly-intact animal, swimming was expressed spontaneously (Fig. 3-3A-C) in the absence of cephalic information. In fact, individual episodes of swimming were extended in the absence of descending input (Fig. 3-3C). Using the reversible sucrose block, our data reconfirm previous conclusions that the cephalic ganglion plays a largely inhibitory role over the decision to swim (Brodfuehrer and Friesen, 1986a; Brodfuehrer and Burns, 1995; Cornford et al., 2006). Once activated, the dynamics of swimming are fairly stereotypical and can continue with strong intersegmental coordination independent of cephalic input (Kristan and Calabrese, 1976).

Although the leech does possess gating cells for swimming, they are distributed among the segmental ganglia housing the swim oscillators. The most prominent of these swim gating cells is cell 204, which projects through Faivre's nerve (Weeks and Kristan, 1978). By severing this nerve, we showed that cell 204 is not needed to gate crawling (Fig. 3-9A). The segmental distribution of cell 204, in combination with a series of tightly coupled oscillators, may help to explain why swimming is less dependent on higher-order descending control. Because the swim circuitry has already been studied

in exquisite detail (reviewed in: Brodfuehrer et al., 1995; Kristan et al., 2005), future studies of crawling may reveal functional architectures optimized for variable (slow) vs stereotypic (fast) forms of locomotion, features likely conserved across animal groups.

Unlike the 'trigger' neurons for swimming (Brodfuehrer and Friesen, 1986b), crawl-activating cells in the cephalic ganglion express gating properties; their activity must be sustained for crawl cycles to be repeated (Esch et al., 2002). In addition to providing the coordinating signal, on-going cephalic activity might also route sensory and efferent signals vital for postural or reflexive adjustments during crawling (Mesce et al., 2008). Although we did not focus on the effects of sensory systems to entrain or reset the crawl oscillators, our preliminary data indicate that mechanosensory inputs can alter their timing (Puhl and Mesce, unpublished observations). In the stick insect, sensory signals from stepping front legs can entrain the on-going stepping of middle and back legs; however, local load signals in middle and hind legs can override this synchronization (Borgmann et al., 2009). Regardless of the signal source (i.e., sensory or cephalic signals), important parallels emerge: the intersegmental activation and coordination of segmental oscillators in tandem with local modification enable an animal to overlay an array of movements or searching-related behaviors while locomoting; all performed without missing a step.

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Figures

Figure 3-1: Effectiveness of the sucrose block during electrical stimulation (black bars under traces). **A**, Schematic diagram of the two-ganglion (M3-M4) preparation. To block neural signals in the connectives, saline in the bowtie-shaped well was replaced with an isotonic sucrose solution. Reintroduction of saline in the well restored neural transmission. Connectives anterior to M3 were stimulated for 1 s using a suction electrode. Extracellular unit activity was recorded in the connectives posterior to M4. **B**, Baseline connective recording prior to stimulation. **C**, Unit activity in response to electrical stimulation prior to the block (Pre). **D**, Recording obtained during the block (Blocked). **E**, Return of electrically-induced activity after removal of the block (Post).

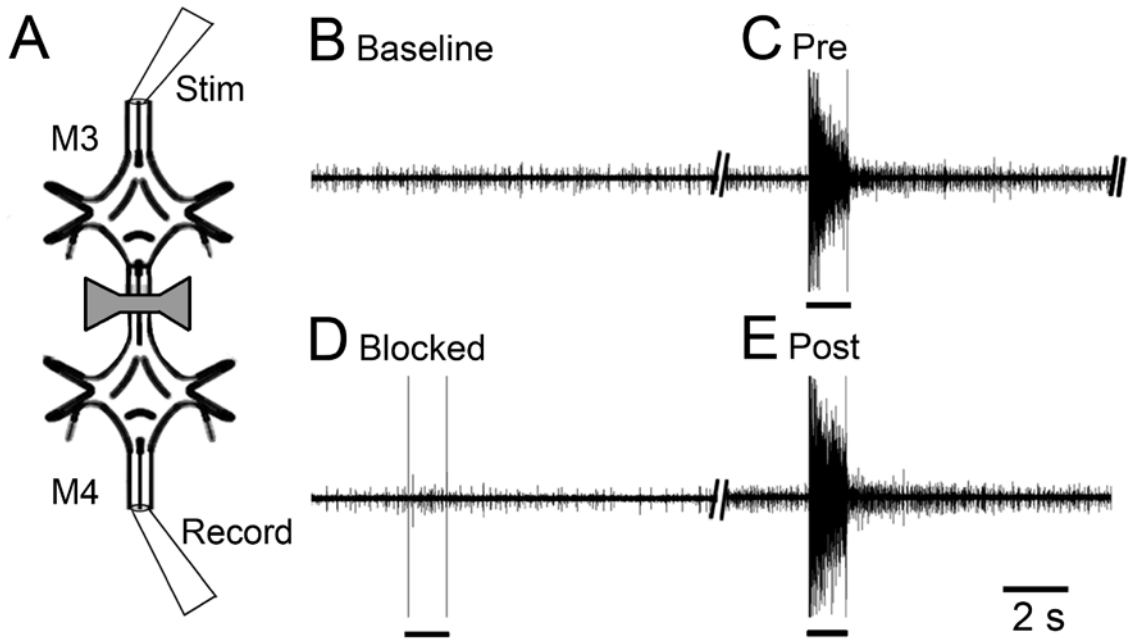


Figure 3-1: Effectiveness of the sucrose block during electrical stimulation.

Figure 3-2: Crawling behavior in the nearly-intact leech and its dependence on descending signals from the cephalic ganglion. A, Schematic drawing of the nearly-intact leech preparation. A bowtie-shaped well was placed across the connectives between M2-M3. The well was sequentially filled with saline, isotonic sucrose, and then saline to restore neural transmission. B, Schematic drawing of overt crawling behavior in nearly-intact leech preparations when descending signals were not blocked. Numbers i.-iii. indicate the contraction phase and iv.-vi. the elongation phase of one overt crawl cycle. C, Number of spontaneous crawl cycles observed for 8 animals during a 5-min interval for each condition (i.e., Pre-Block, Blocked, Post-Block). D, Number of trials (out of 5) that crawling was elicited in 8 animals after a gentle hyper-elongation of the body; same three conditions were tested. C,D, Black bars represent shallow water (< 1 mm depth) and grey bars represent deep water (10 mm depth). ** = $p < 0.01$. Error bars are \pm SEM.

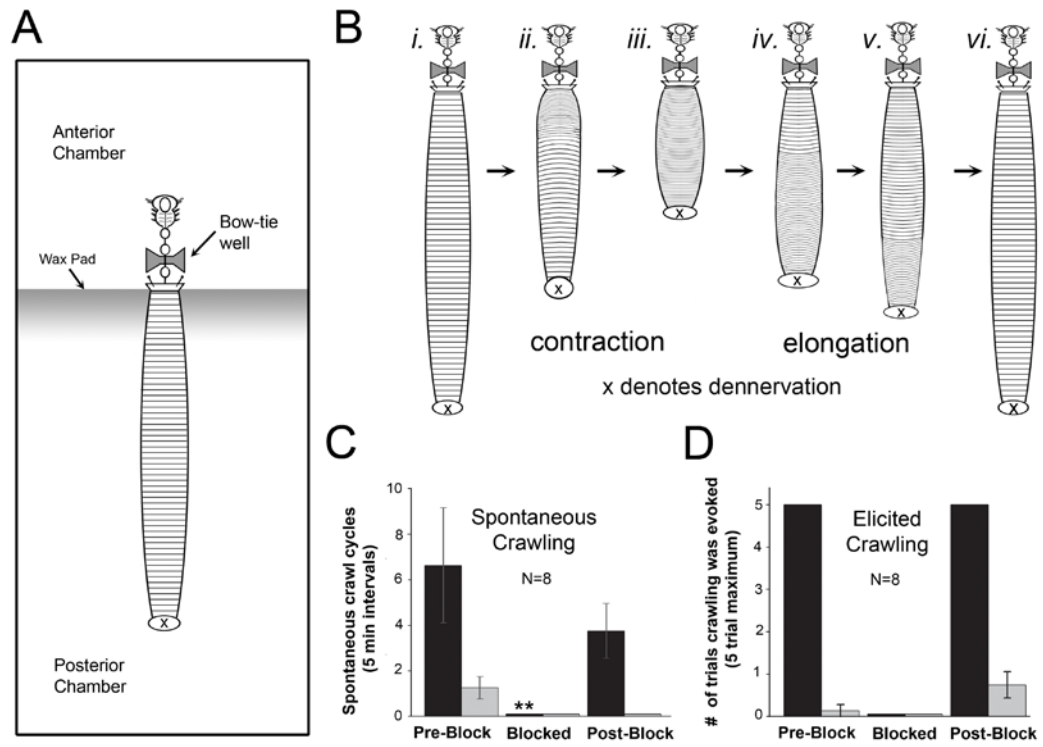


Figure 3-2: Crawling behavior in the nearly-intact leech and its dependence on descending signals from the cephalic ganglion.

Figure 3-3: Swimming behavior in the nearly-intact leech and its lack of dependence on descending signals. **A**, Number of spontaneous swim episodes (uninterrupted bouts of swimming) in a 5-min interval for each condition (Pre-Block, Blocked, Post-Block). Overt swim bouts were easily discerned as the animal did not pause between successive swim cycles. **B**, Total number of spontaneous swim cycles in a 5-min interval for each experimental condition. **C**, Number of spontaneous cycles per swim episode for each experimental condition. **D**, Number of trials (out of 5) in which swimming was elicited after gently stroking the dorsal body surface. All bars represent swimming in high water conditions (10 mm depth). No leeches swam in low water (< 1 mm depth). * = $p < 0.05$, ** = $p < 0.01$. Error bars are \pm SEM. $n = 8$ leeches.

Swimming in Nearly-Intact Preparations (N=8)

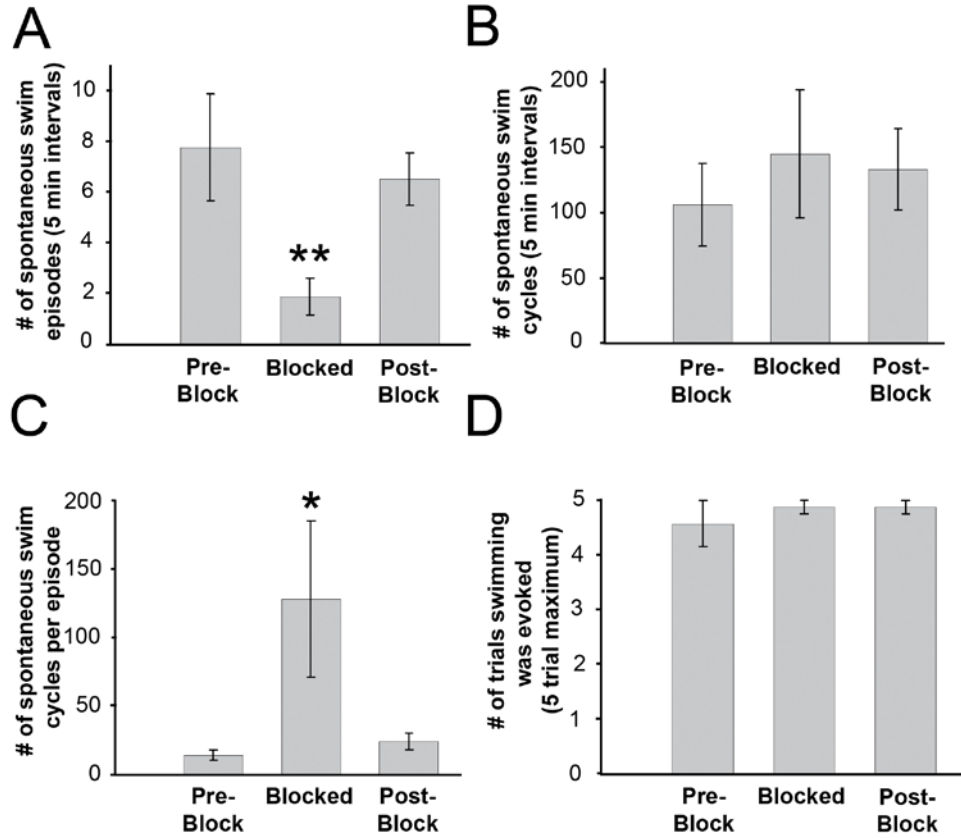


Figure 3-3: Swimming behavior in the nearly-intact leech and its lack of dependence on descending signals.

Figure 3-4: Cephalic descending signals are necessary and sufficient for appropriate intersegmental coordination during DA-biased fictive crawling. **A**, Schematic drawing of the whole nerve cord and placement of the bowtie-shaped well containing saline (Block OFF) or isotonic sucrose (Block ON). Locations of extracellular recordings are marked by electrodes on 3 evenly spaced dorsal posterior (DP) nerves (M10, M12 and M14). Site of K^+ excitation is indicated at the left of the cephalic ganglion. **B**, Initial record shows DP nerve activity during blockade of cephalic signals but in the presence of cephalic K^+ excitation (vertical arrow). The largest unit in the DP recordings is from the dorsal longitudinal excitator motoneuron DE-3, which is active during the contraction phase of crawling (see below). When the block was removed (vertical dotted line), coordinated fictive crawling emerged. **C**, DP nerve activity from the same preparation after DA-biased crawling had subsided (initial traces). When the cephalic ganglion was briefly stimulated by K^+ (vertical arrow), robust and coordinated fictive crawling ensued. A representative crawl period is highlighted with text, and the fictive elongation (E) and contraction (C) phases of crawling (2 cycles) are shown for the sake of clarity. **D**, Expanded trace of DP nerve activity when the sucrose block was in effect. Previous crawling exhibited prior to this block (**C**) was replaced with uncoordinated DE-3 bursting.

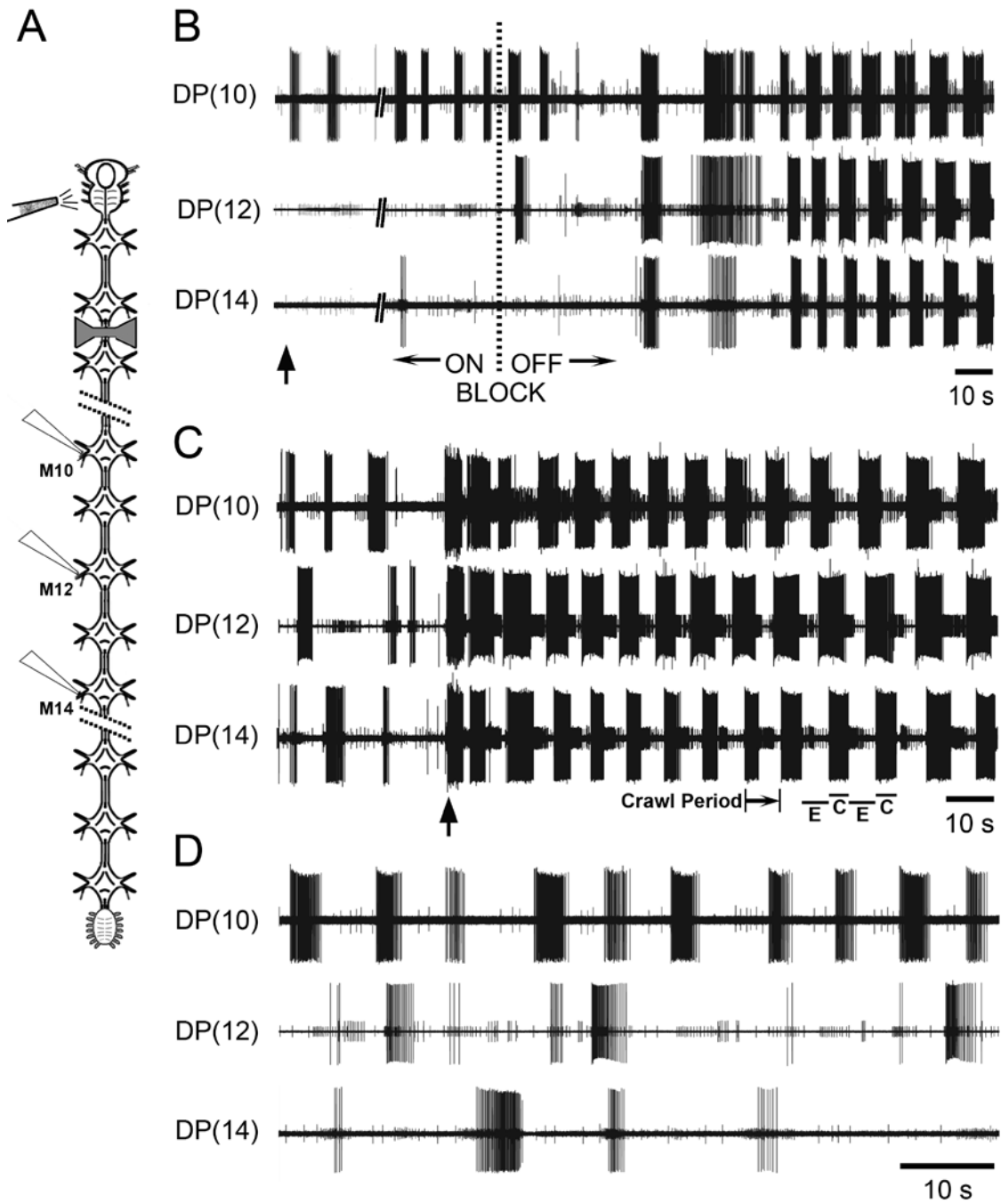


Figure 3-4: Cephalic descending signals are necessary and sufficient for appropriate intersegmental coordination during DA-biased fictive crawling.

Figure 3-5: In whole isolated nerve cords, sucrose blockade of cephalic descending signals abolishes the posterior-directed intersegmental coordination of the DA-biased crawl oscillators. **A**, The number of preparations that stopped crawling (SC) and those that had altered coordination during the sucrose block. **B**, DP recordings of fictive crawling in two adjacent segments prior to the block (Pre-Block) and during it (Blocked). Intersegmental delays (ISDs) were altered during cephalic blockade and intersegmental phase advances (asterisks) were became evident (note: rare in controls). **C**, DP nerve activity from 3 segments showed robust and coordinated fictive crawling in Pre-Block (left). Shortly after blocking descending inputs (Blocked, right traces), uncoordinated DE-3 bursting emerged; this preparation exemplifies those with variable periods. **D**, Boxplots (black = Pre-Block; white = Blocked) summarizing changes in DE-3 intersegmental phase delays for preparations showing no significant change in cycle period ($n = 9$, labeled i.-ix.). Plus symbols denote outlier data points. **E**, Fictive crawl periods obtained from 3 segmental oscillators plotted over successive cycles in Pre-Block and again when cephalic inputs were removed (Blocked) ($n = 3$ whole nerve cord preparations labeled i.-iii.). Prior to the block (plots at left), the outputs from all 3 oscillators exhibited the same period. During the blockade (plots at right) periods were no longer stable and varied across all 3 segments. Data for cycle periods during the block were obtained within the following time windows: 74 s (i), 158 s (ii) and 381 s (iii); thus some cycles have only 1 or 2 symbols shown.

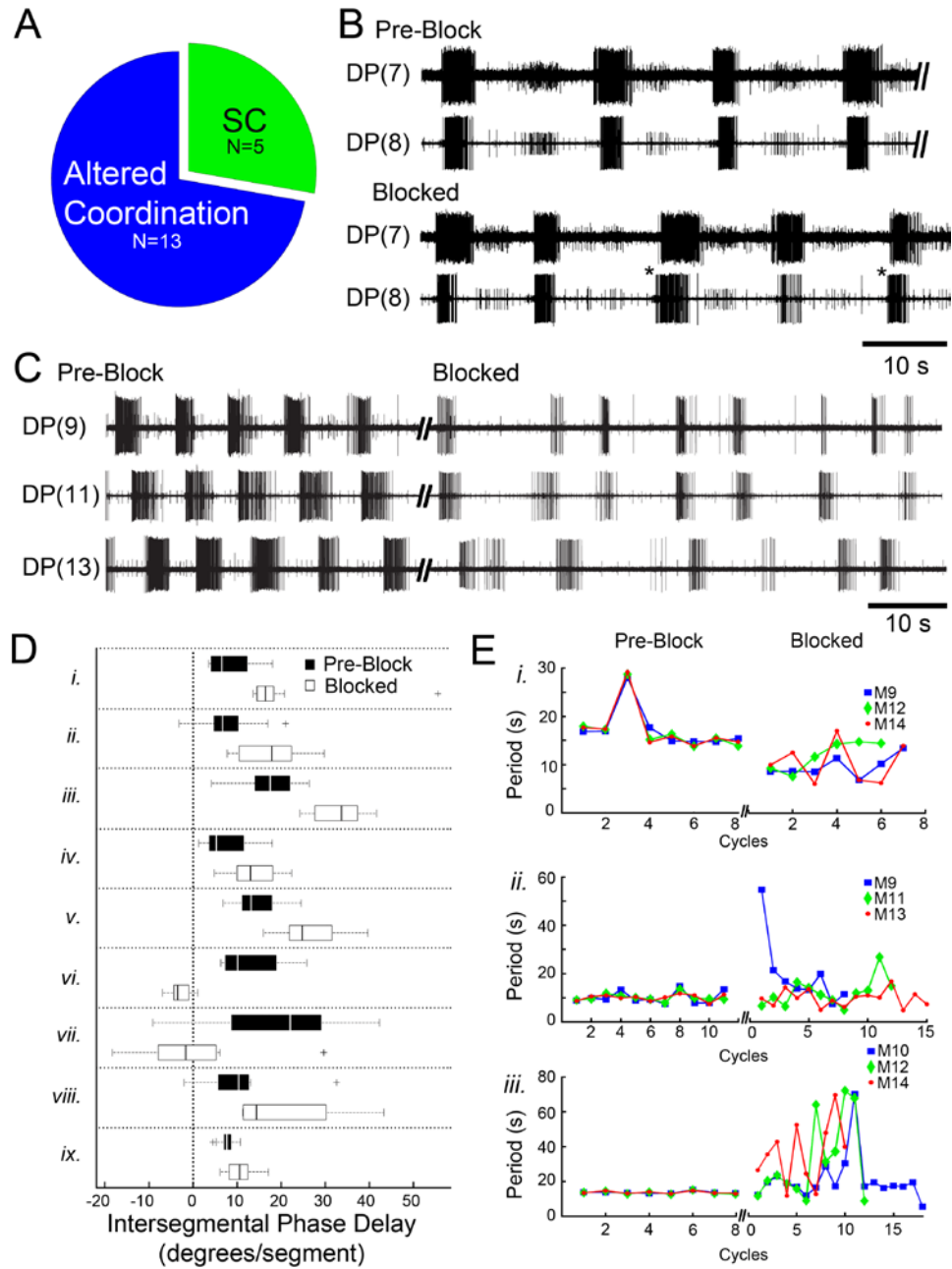


Figure 3-5: In whole nerve cords, blockade of cephalic descending signals abolishes intersegmental coordination of the DA-biased crawl oscillators.

Figure 3-6: In the absence of cephalic inputs, a chain of DA-biased ganglia reveals uncoordinated and unstable DE-3 bursting, but a single ganglion isolated from this chain transitions to stable and robust fictive crawling. **A**, Schematic of a chain of 5 ganglia (M8-M12) (left) and motor activity recorded from DP nerves 8,10,12 (right). Recordings revealed the lack of robust rhythmic DE-3 bursting and showed an inappropriate metachronal wave of intersegmental DE-3 activity. **B**, Left, schematic showing the physical isolation of one ganglion (M8) from the chain. Right, DP nerve activity from the same preparation as in (**A**) 4.7 min after transecting the M8-M9 connectives. DE-3 bursting in the isolated ganglion became more robust and burst structure solidified; unstable bursting activity in DP(10) continued. **C**, DP recordings 8 min after transection showing the emergence of robust fictive crawling [DP(8)] with stable shorter crawl periods (top trace). DE-3 bursting in the other two ganglia was absent (middle and bottom traces).

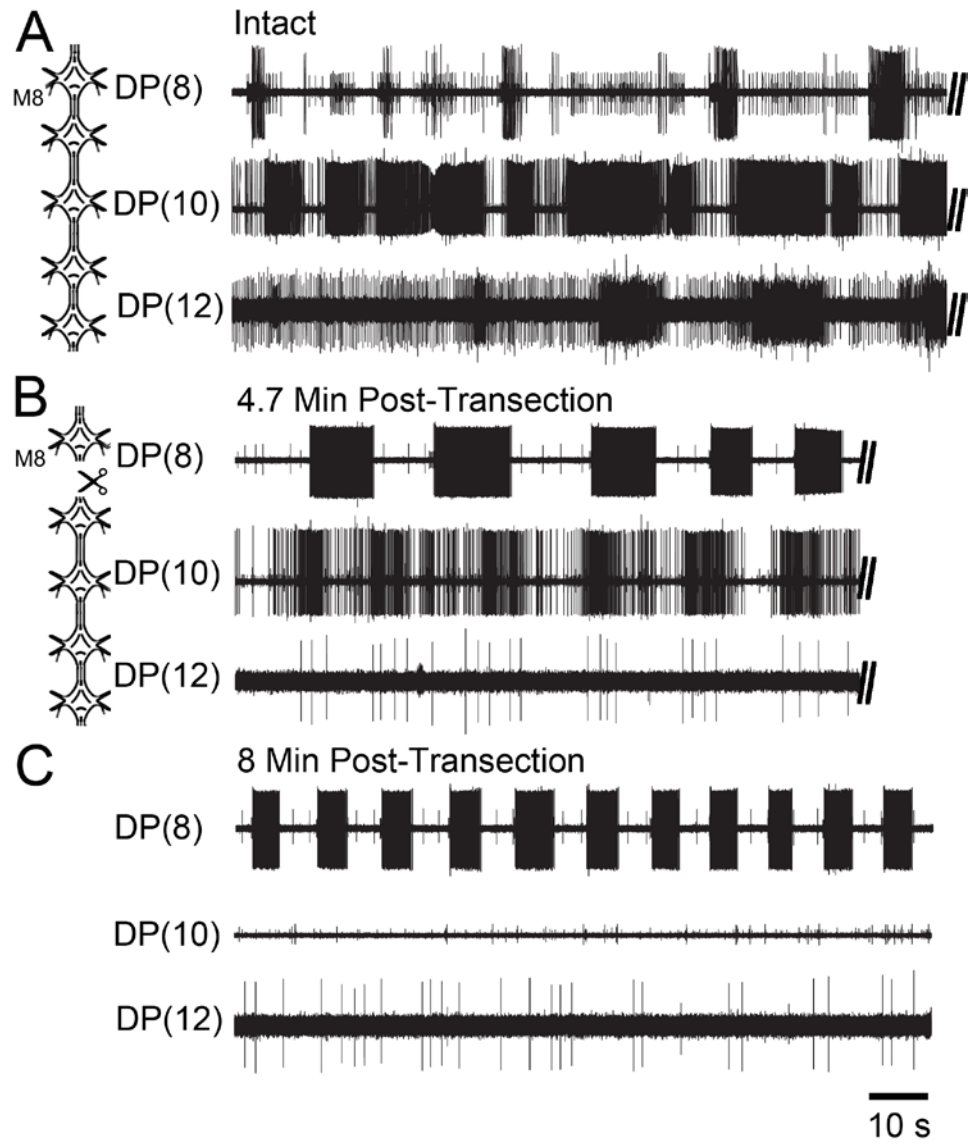


Figure 3-6: In the absence of cephalic inputs, a chain of DA-biased ganglia reveals uncoordinated and unstable DE-3 bursting.

Figure 3-7: Ability of a single DA-activated crawl oscillator to drive crawl-like activity in adjacent ganglia. **A-C**, Left, schematic drawings indicating site of DA placement (shaded well; 100 μ M DA) and specific ganglia recorded. Shaded records correspond only to those ganglia treated with DA. **A**, The DA-treated ganglion (G/DA, middle trace) expressed fictive crawling, which promoted crawl-like DE-3 bursting activity (dotted lines) in the adjacent anterior ganglion (G/-1, top trace) and posterior ganglion (G/+1, bottom trace). **B**, DA-induced fictive crawling [upper trace, DP(9)] caused the untreated posterior ganglion [bottom trace, DP(10)] to show DE-3 burst-like activity. Furthermore, the intracellular record of the circular MN CV (middle trace) indicates that it is hyperpolarized (dotted lines) in phase with DE-3 excitation (bottom trace); an indicator of true fictive crawling. **C**, True fictive crawling, although notably quite weak, was also observed in the anterior ganglion (top and middle traces) in response to DA placed on its posterior neighbor (bottom trace); here CV was hyperpolarized (top trace, dotted lines) in phase with DE-3's depolarization (middle trace). **D**, Percentage of preparations exhibiting DE-3 bursting in untreated ganglia located adjacent to ganglia induced to crawl by DA. **E**, Percentage of preparations exhibiting anti-phasic activity in CV and DE-3 motoneurons. **F**, Fictive crawl-like periods (i.e., DE-3 bursts) were similar across untreated ganglia located anterior (G/-1) and posterior (G/+1) to a given DA-treated ganglion (G/DA). **G**, Number of DE-3 spikes/burst across untreated and DA-treated ganglia (G/-1, G/DA, G/+1). Note that the number of DE-3 spikes/burst between G/-1 and G/+1 is very different. * = $p < 0.05$. Error bars depict \pm the SEM. Numbers inside the bars of **D-G** denote the sample sizes.

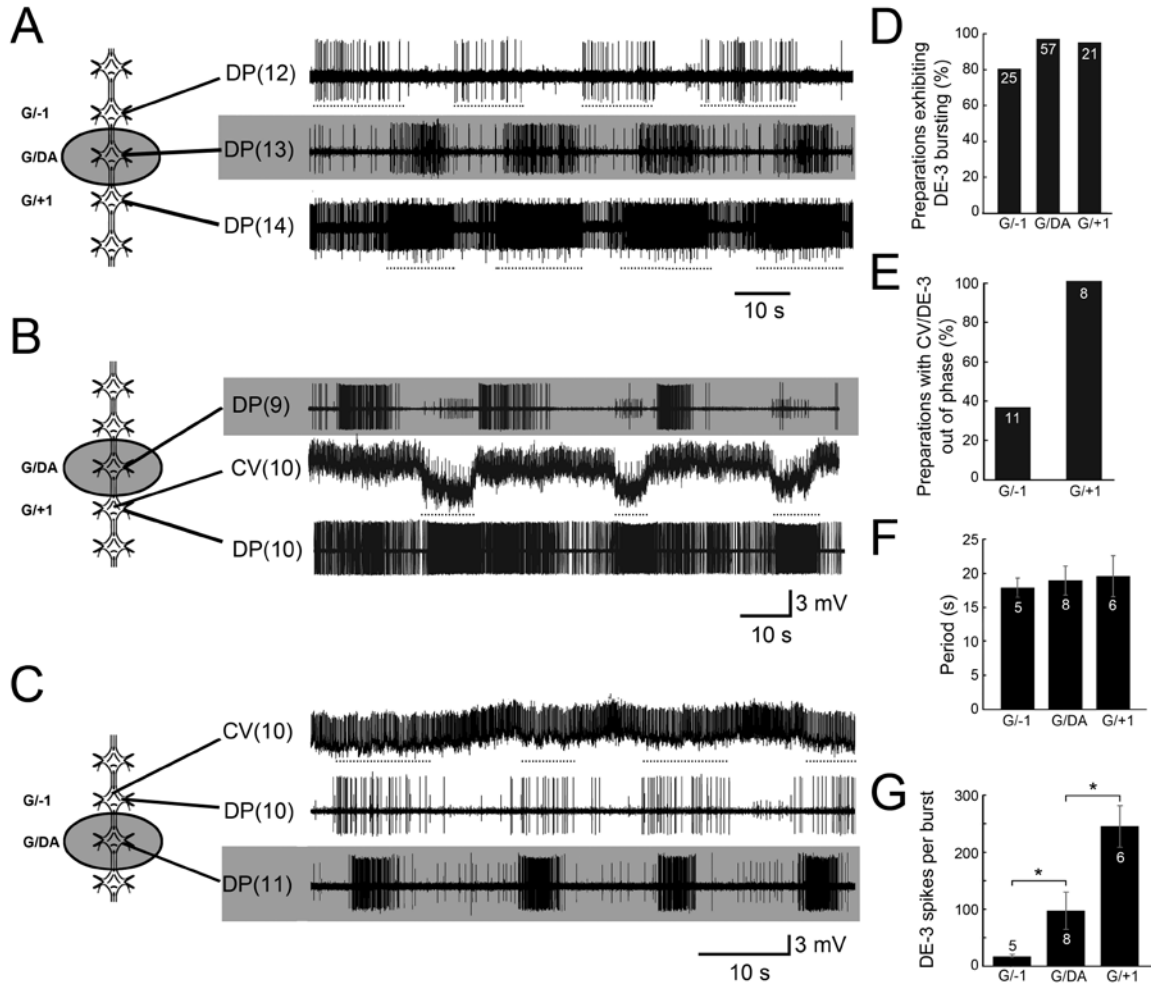


Figure 3-7: Ability of a single DA-activated crawl oscillator to drive crawl-like activity in adjacent ganglia.

Figure 3-8: Crawl-like drive from an individual crawl oscillator extends to multiple posterior ganglia; signals can be carried via fibers of passage spanning two ganglia away from their source. **A,C,D**, Left, schematic drawings indicating site of DA placement (shaded well; 100 μ M DA) and specific ganglia recorded. Shaded recording traces correspond only to those ganglia treated with DA. **A**, Fictive crawling in the DA-treated ganglion (top trace) and crawl-like activity driven in untreated ganglia up to two segments away (middle and bottom traces). **B**, Percentage of preparations exhibiting DE-3 bursting in response to DA on a given single ganglion (G/DA); drive extended up to 3 segments away (G/+3). Numbers inside bars denote sample sizes. **C**, Ganglia showing tonic DE-3 activity across 3 segments [DP(9, 10, 11)] prior to treatment. **D**, DP recordings from the same preparation as in (**C**) after DA-activated fictive crawling was induced (top trace) but chemical synaptic transmission was blocked in the adjacent posterior ganglion (middle trace). Box (left) surrounding ganglion M10 (\uparrow Mg⁺⁺/ \downarrow Ca⁺⁺) indicates procedure that resulted in no unit activity in DP(10) (middle trace). As shown in the DP(11) recording (bottom trace), DE-3 bursting was present, although weak, thus indicating that some crawl-like drive extended past this silenced ganglion.

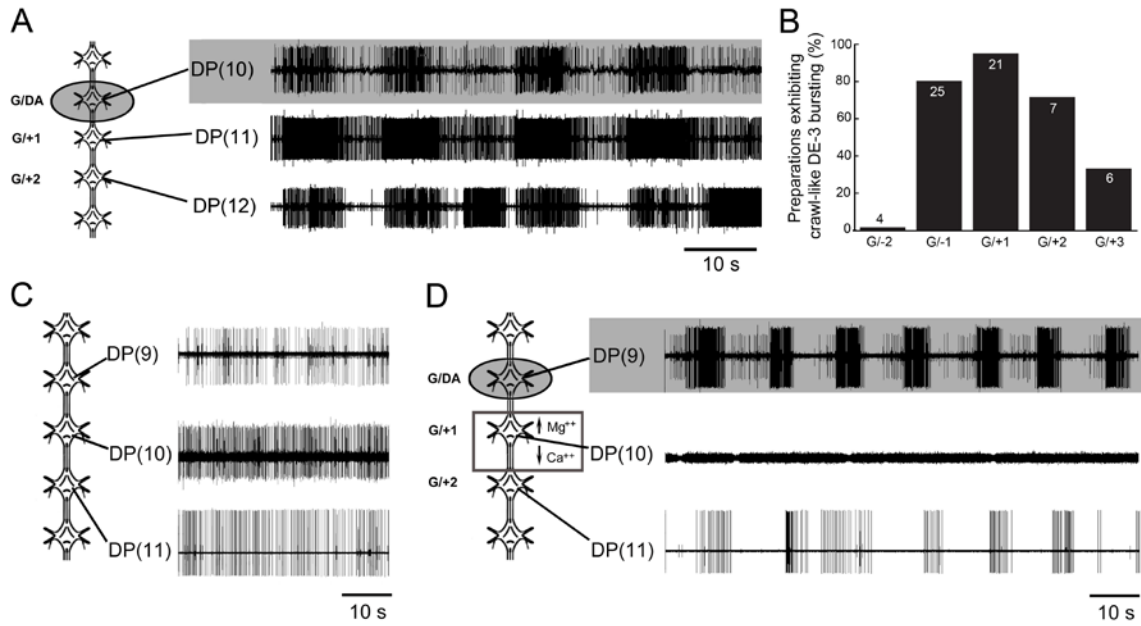


Figure 3-8: Crawl-like drive from an individual crawl oscillator extends to multiple posterior ganglia.

Figure 3-9: Testing the role of Faivre's nerve (FN) and the hemi-connectives in transmitting crawl-like drive to adjacent ganglia. **A**, Left, schematic drawing indicating site of DA placement (shaded well; 100 μ M DA), specific ganglia recorded and location of cut Faivre's nerve. Right, shaded recording corresponds to ganglion in DA (upper trace) that exhibited robust fictive crawling [DP(16)]. Bottom trace shows crawl-like activity in adjacent posterior ganglion [DP(17)] after selective transection of the FN. **B**, Left, schematic marking location of cut hemiconnectives. Right, upper trace shows DA-induced fictive crawling [DP(9)]. Bottom trace reveals the absence of any rhythmic DE-3 activity as a result of transecting the hemi-connectives. **C**, Percentage of preparations in which G/+1 exhibited fictive crawl-like bursting when the FN was cut (left bar) and when the hemi-connectives were cut (right bar). Sample sizes are denoted for each bar.

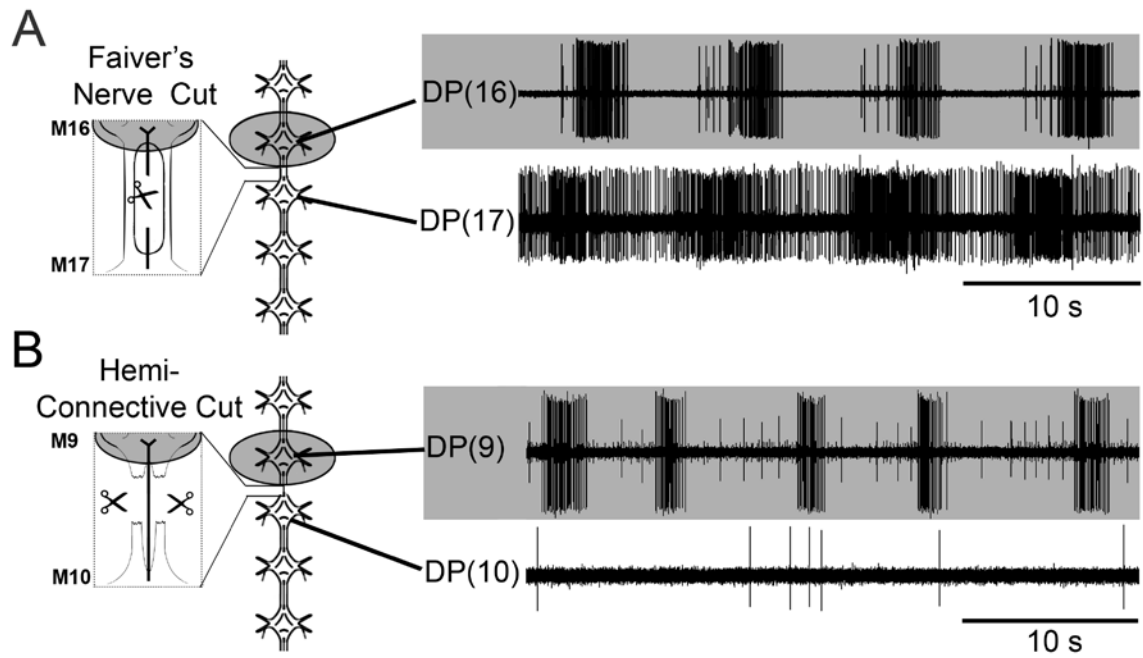


Figure 3-9: Crawl-like drive is transmitted via the hemi-connectives.

Figure 3-10: Schematic diagram of the leech CNS depicting neural elements involved in the intersegmental coordination of crawling. Each of the 21 midbody ganglia contains a complete crawl oscillator (circle with sigmoid) (Puhl and Mesce, 2008). The descending long-distance coordination signal(s) from the cephalic ganglion is necessary and sufficient for the production of normal intersegmental phase delays between the oscillators (continuous black line with lateral arrows). In addition, individual crawl oscillators provide weak drive (open arrows) to their anterior neighbor and much stronger excitatory drive (thick gray arrows) to two or more oscillators in posterior locations. Intersegmental fibers of passage delivering crawl-like drive can project uninterrupted at least two ganglia away from their source (thinner gray arrows).

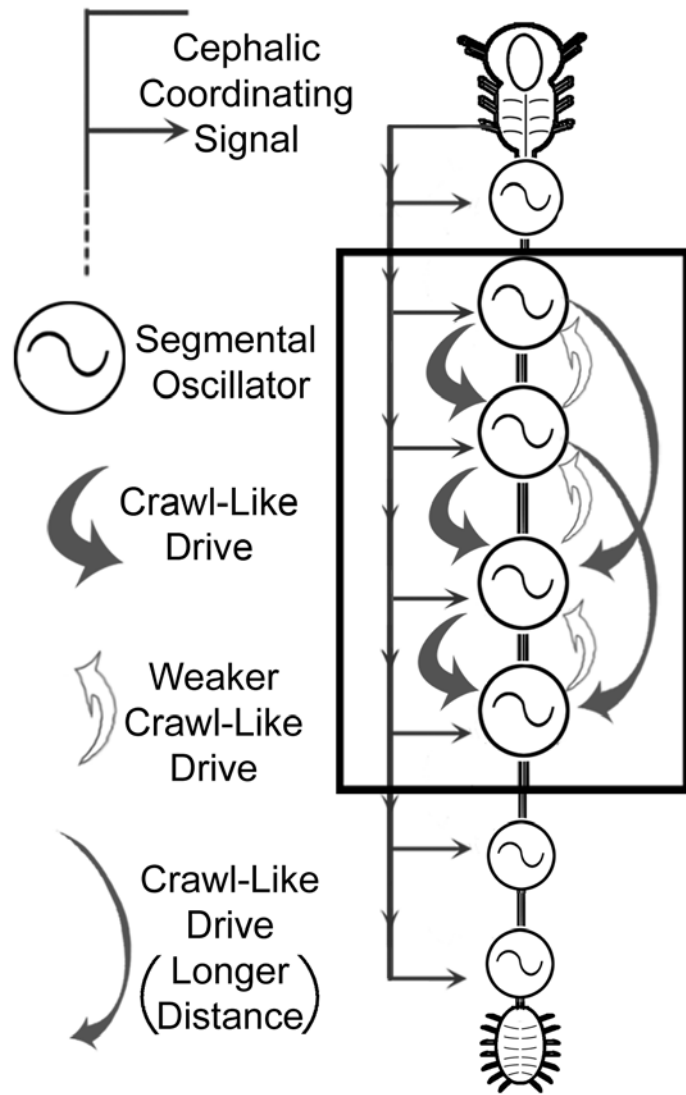


Figure 3-10: Model of intersegmental coordination for crawling.

Chapter Four:

Necessary, sufficient and permissive: A single locomotor command
neuron responsible for intersegmental coordination

In this report we posed the over-arching question: What contributions can a single neuron have on controlling the behavior of an animal? To address this important question, we studied R3b-1, a long-distance projecting neuron that is bilaterally paired and located in the cephalic ganglion of the medicinal leech. We established that this interneuron has the cytoarchitecture to communicate potentially with both left and right halves of each segmental ganglion within the central nervous system (CNS). We also determined that R3b-1 activity is particularly important for the expression of fictive crawling, as spiking in a single R3b-1 was determined to be both necessary and sufficient for crawling behavior. Aside from its command-like properties, R3b-1 was found to modulate the frequency of crawl-related motor outputs; furthermore, when the CNS was biased to crawl by exposure to DA, crawling became the exclusive locomotor pattern produced by R3b-1 as opposed to a mixture of crawling and swimming outputs. Although the above attributes render R3b-1 an exceptional cell, it is its ability to coordinate the segmentally-distributed crawl oscillators that makes this singular neuron so notable. To our knowledge, this cell provides the first biological example of a single command-like neuron that is also vital for the intersegmental coordination of a locomotor behavior.

Introduction

Higher-order neurons that control specific behavioral routines must take into account a variety of conditions, such as the environmental and internal state of the organism (i.e., the context). The current consensus is that most animals employ an array of different strategies and decision-making neurons to help sort through the many factors involved in selecting a given behavior to express (Kupfermann and Weiss, 2001; Ganguli et al., 2008; Kristan, 2008). Historically, studies have focused on the population-level influences that decision-making systems have on behaviors (Platt, 2002; Pesaran, 2010). Others have investigated how single cells or populations of homologous cells influence an animal's behavior and have focused on a single dimension of a single behavior (Wiersma and Ikeda, 1964; Larimer et al., 1971; Davis and Kennedy, 1972; Gillette and Davis, 1977). A growing number of reports, however, have provided evidence that even single neurons can influence more than one aspect of a behavior (Böhm and Schildberger, 1992; Hedwig, 2000), and can participate in selecting more than one behavior depending on changing contextual cues (Ritzmann et al., 1980; Hooper and Moulins, 1989; Baader, 1997; Esch et al., 2002; Briggman et al., 2005; Briggman and Kristan, 2006; Berkowitz et al., 2010). Thus a fascinating question arises: What contributions does a single neuron fully make in selecting and controlling behaviors? Answering this question is often daunting in vertebrate animals because of the sheer number of cells and complexity of behavioral routines expressed. Studying behaviors having quantifiable and stereotypical outputs, such as locomotion, reduces this difficulty, as does studying simpler invertebrate animals that possess a reduced number of neurons which are easily accessible and identifiable as individuals.

In this study, we used the medicinal leech to investigate the breadth of contributions that a single higher-order neuron can have on locomotor-related behaviors. Our aim was to understand the multi-dimensional roles played by a cephalic neuron, R3b-1, and how contextual features alter these features. Cell R3b-1 was shown previously to activate crawling behavior, which is the primary form of terrestrial locomotion for leeches (Esch et al., 2002). [Crawling is defined as the alternating elongation and contraction of the body with coordinated sucker attachments; segmental body movements travel exclusively as a posteriorly-directed metachronal wave (Gray et al., 1938)].

Cell R3b-1 is clearly involved in the decision to crawl, yet it also participates in the decision to swim if contextual conditions indicate an aquatic-like environment. Furthermore, R3b-1 projects its axon uninterrupted along the entire length of the CNS (Esch et al., 2002), thus it has direct access to each of the 21 segmental ganglia comprising the CNS. Importantly, each segmental ganglion has been shown to contain a complete set of rhythm-generating elements (i.e., central pattern generator, CPG) for crawling (Puhl and Mesce, 2008), thus opening up the possibility that R3b-1 may also function as an intersegmental coordinating cell and be an important element for the timing aspects of the crawl motor pattern. Because each of the crawl CPGs or unit burst generators is activated by dopamine (Puhl and Mesce, 2008), this finding begs the question of whether R3b-1 is also susceptible to modulation by dopamine and posits whether such an influence can sculpt R3b-1's activity to facilitate crawling.

In this report, we demonstrate that cell R3b-1 is, indeed, a command neuron for crawling, fulfilling the criteria of necessity and sufficiency first put forth by Kupferman and Weiss (1978). Since this landmark perspective, however, the idea of the command or command-like neuron has undergone a number of revisions to incorporate the

importance of context or state-dependency (Kupferman and Weiss, 2001). In addition, individual command-like neurons or populations of cells can now be viewed as having more of a permissive or gating effect over behavior if conditions are right. The multiple executive functions and other attributes of cell R3b-1 discovered in the current study underscore that even when a classical command neuron is established, as few have been, the dimensional breath of such cells are not fully unveiled unless the contextual state of the animal is varied.

Materials and Methods

Animals and physiological solutions

Adult medicinal leeches (*Hirudo verbana*) between 1.5 - 3.0 g in weight were obtained from Niagara Medical Leeches (Niagara Falls, NY, USA) and maintained at room temperature in pond water (distilled water containing dilute Instant Ocean sea salts, 1.89 g/gallon; Spectrum Brands).

Prior to dissection, leeches were anesthetized on ice. Dissections and all physiological recordings were performed at room temperature in normal leech saline. Normal leech saline consisted of (in mM): 116.0 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.5 MgCl₂, 10.0 dextrose, and 10.0 Trizma preset crystals, pH 7.4 (Sigma-Aldrich) (adapted from Nicholls and Baylor, 1968). High magnesium/low calcium saline (20:1) used to block synaptic transmission contained (in mM): 87.0 NaCl, 4.0 KCl, 1 CaCl₂, 20 MgCl₂, 10.0 dextrose, and 10.0 Trizma preset crystals, pH 7.4 (Szczupak et al., 1998). Dopamine-containing stock saline (1 mM) was prepared fresh daily and used within 5 h. Final DA concentrations of 50-100 µM for experimental use were prepared by diluting the stock with normal leech saline (Puhl and Mesce, 2008).

Leech neuroanatomy and terminology

The central nervous system (CNS) of the medicinal leech comprises a compound cephalic ganglion [i.e., fused supraesophageal and subesophageal ganglia], 21 midbody ganglia (one per body segment designated M1-M21), and a compound tail ganglion (schematic; Fig. 4-3A).

The subesophageal ganglion (SEG) is divided into 4 neuromeres named R1-R4, each containing a dorsal and ventral layer of neuronal somata (Esch et al., 2002). The dorsal sides of R2-R4 are sub-divided further into 'a' and 'b' sub-packets. Cell R3b-1, a previously identified descending projection neuron that is involved in locomotion (Esch et al., 2002; Mesce et al., 2008) is thus located in the second sub-packet of the third neuromere (R3b).

For this study we monitored the activity of two uniquely identifiable motoneurons (MNs) active during crawling, the dorsal longitudinal excitor 3 (DE-3) MN and the ventral lateral circular excitor (CV) MN located on the dorsal and ventral surfaces, respectively (Stuart, 1970). CV activity was monitored *via* intracellular electrophysiological recording and DE-3 by extracellular recording of the dorsal posterior (DP) nerve root, which contains the large DE-3 axon and thus most prominent extracellular spike (Kristan et al., 1974).

Experimental preparations, electrophysiological recordings and cell identification

We removed the complete (i.e., intact) CNS from the leech body with both left and right DP nerves attached in at least 3-5 segmental ganglia. This preparation is referred to as the 'whole CNS preparation' below. Fictive motor outputs were monitored by recording the activity of DE-3 extracellularly *via* the DP nerve. When appropriate, a Vaseline well was placed around 3 continuous segmental ganglia for focal application of

the high magnesium/low calcium saline to block local synaptic activity; this configuration allowed the propagation of activity in fibers of passage (i.e. R3b-1) (Tschuluun et al., 2001). To induce fictive crawling, 50-100 μ M DA was superfused onto the entire preparation using a peristaltic pump (Fisher Scientific). After DA perfusion and prior to observing coordinated fictive crawling (that propagated along the nerve cord from anterior-to-posterior) we sometimes would observe crawl-like bursting in the DP nerves that was not coordinated. During other times, DA-induced coordinated fictive crawling would not emerge until shortly after washing the DA from the preparation with normal saline (Puhl and Mesce, 2008; Puhl and Mesce, 2010).

Intracellular recordings of R3b-1 and CV (singly or simultaneously) were performed using an Axon Instruments AxoClamp 700B amplifier in current clamp mode. Intracellular pipettes were filled with a solution of 2 M potassium acetate containing 20 mM potassium chloride and had resistances of 30-60 M Ω . Electrode tips were filled with 2 M potassium acetate containing 5% (w/v) Neurobiotin (Vector Laboratories). Extracellular multi-unit recordings from 2-3 DP nerves were performed using suction electrodes (~50 μ m tip diameter). Extracellular activity from the connectives was recorded *en-passant* via a suction electrode (~200 μ m tip diameter) so as not to damage the connective's fibers. Extracellular signals were amplified using an A-M Systems 4-channel differential AC-coupled amplifier. Both intracellular and extracellular recordings were digitized using a Digidata 16-channel data acquisition system (Axon Instruments) attached to a Microsoft Windows-compatible personal computer at 10,000 samples/s. Extracellular signals were low-pass filtered at 1 kHz. Electrophysiological signals were visualized and plotted using pClamp (Axon Instruments), Matlab (The Mathworks, Inc.), and Adobe Photoshop CS4.

In whole nerve cord preparations, R3b-1 was identified initially by the position and size of its soma, the existence of a correlated unit observed in the recorded connectives, as well as the emergence of either fictive swimming or crawling in response to 3-4 nA intracellular current injection (Esch et al., 2002). In isolated cephalic ganglion preparations (i.e., separated from the nerve cord between the SEG and M1), we first identified R3b-1 using the criteria described above, but then removed the intracellular electrode, severed the connectives between the SEG and M1 and re-impaled the neuron.

At the conclusion of each experiment the recorded R3b-1 candidate's identity was confirmed by ionophoretically filling the cell with Neurobiotin for 10-60 minutes to elucidate the cell's morphology. Neurobiotin was visualized by fixing and washing the tissue, then treating it with 1:50 Cy3-conjugated Streptavidin in PBS for 24-48 h at 4°C. The tissue was prepared for viewing as described by Gilchrist et al. (1995). We visualized the Cy3 fluorescence using a Quantum 512SC monochrome digital camera attached to an Olympus BX61 research microscope with disc scanning confocal unit (DSU) capabilities. Images were collected using Metamorph 7 and prepared for publication in Adobe Photoshop CS4.

Dual hemi-connective transections and observations of overt crawling in intact leeches

We monitored crawling behaviors in intact leeches before and after dual ipsilateral hemi-connective transections above and below M2 (schematic Fig. 4-4A). Prior to and during surgery leeches were placed on ice. A transverse incision (5-10 mm) was made over the vicinity of M2 and a ventral longitudinal slit was made to the ventral blood sinus above and below M2. Ipsilateral hemi-connective transections above and below M2 were made using fine scissors. After surgery, leeches were placed in plastic

containers filled with pond water. Although we did not perform any wound-closing procedures, bleeding after surgery stopped within 30 minutes. Sham surgeries were performed as described, but without severing the hemi-connectives.

We video recorded spontaneous crawling behaviors of transected and sham controls one day prior to (day -1) and for several weeks after surgery. Behaviors were monitored for 8 minutes in a circular plastic arena (25 cm diameter) containing ~ 20 mL of pond water to wet the bottom surface of the arena. The number of crawl cycles during each observation period was determined for each animal as was the start and end times of the elongations and contractions for 5 consecutive crawl cycles.

Data analysis

To calculate the temporal characteristics of R3b-1 bursting, we determined the end time of 6-10 consecutive R3b-1 bursts (which signified the end of elongation and the beginning of the contraction phases of crawling (Fig. 4-6; Esch et al., 2002). We demarcated the end times of R3b-1 bursts as the time when R3b-1 spiking ceased and the membrane potential became more hyperpolarized (Fig 4-6, arrows). To determine the temporal characteristics of the observed fictive motor patterns, we analyzed the bursting activity of DE-3, the largest unit in our DP recordings (see above). Start times for each of 6-10 consecutive DE-3 bursts were readily discernable by visual inspection in pClamp. A grouping of DE-3 spikes was deemed to be a burst if at least 10 spikes occurred with an inter-spike interval of <2 s, however, the vast majority of bursts contained dozens of spikes with a higher rate of DE-3 spiking (> 2 Hz). The period (p) of bursting was calculated as the time interval between the start of consecutive DE-3 bursts. Mean periods were calculated using 6-10 consecutive bursts. The frequency (f) of fictive crawling was calculated as the reciprocal of the crawl period ($f = 1/p$). Rhythmic

fictive motor patterns with periods shorter than 2 s were deemed to be fictive swimming while those with periods greater than 5 s were deemed to be fictive crawling (Kristan et al., 1974; Puhl and Mesce, 2008). The shortest fictive crawling period we observed was 7 s, thus easily partitioning fictive swimming from crawling. Intersegmental delays (ISDs) between R3b-1 and DE-3 bursting or between DE-3 bursting across segments were determined by measuring the time delay of burst start times (or burst end times for R3b-1) in two segments and dividing by the number of segments that separated the two recordings. Intersegmental phase delays (ϕ) were calculated by dividing the ISD by the corresponding crawl period for that cycle ($\phi = \text{ISD}/p$). The most anterior recording was used as a reference signal for all ISD and phase delay calculations. All mean values are presented \pm the standard error of the mean (SEM).

The firing frequency of R3b-1 was determined by counting the number of spikes observed in the intracellular recording in a 2 s window and dividing by 2 s. If the intracellular trace was not visible, such as during large amplitude current injections, we determined R3b-1 spike rates in the connectives as follows. We used R3b-1 spikes in the intracellular traces as a reference to find the correct unit in the connective recording. A template of the connective spike was then made in Clampfit based on 20-30 R3b-1 action potentials and the template matching functions of Clampfit were used to detect the number of extracellular spikes.

Two-tailed, one-way Student's *t*-tests were performed using the software package R (R Development Core Team, 2010; URL: <http://www.R-project.org>). The confidence level used was 0.95 ($\alpha = 0.05$).

Results

Dopamine biases the output of R3b-1 towards crawling

In nearly-intact leech preparations, electrical stimulation of the cephalic interneuron R3b-1 can elicit overt swimming or crawling depending on whether the body of the leech is suspended in fluid or touching the substrate (Esch et al., 2002). In isolated whole nerve cords, however, Esch et al. (2002) observed that a given preparation did not switch between crawling or swimming and expressed only one form of fictive locomotion, typically crawling. Of 24 isolated whole nerve cords that we tested, 19 exhibited fictive crawling and 5 produced fictive swimming in response to intracellular current injection of R3b-1 (3 - 4 nA; Fig. 4-1A). However, when we subsequently applied 50-100 μ M dopamine (DA) to each of these 24 preparations, all 24 of them expressed fictive crawling upon R3b-1 excitation. Thus the same 5 nerve cords that previously showed swimming exclusively, now produced solely crawling in response to identical current injections (Fig. 4-1A).

Does DA cause a change in the electrical activity of R3b-1 and, if so, might this provide at least a partial explanation for the switch to crawling? To ensure that potential crawl-related ascending inputs from the posterior crawl oscillators might not cloud our results, we recorded from R3b-1 in a preparation consisting of the cephalic ganglion detached from the posterior segmental ganglia (schematic, Fig. 4-1B). Prior to 100 μ M DA application, R3b-1 spiked tonically (N = 4) in isolated cephalic preparations (Fig. 4-1C), but after DA exposure (at time of application or during washout) R3b-1 exhibited slow rhythmic oscillations in its membrane potential with rhythmic modulation of its spiking activity (N=4) (Fig. 4-1D). These large oscillations had a period matching the fictive crawl cycles previously monitored in crawl-related motoneurons during bouts of

DA-induced crawling (Puhl and Mesce, 2008). Because ascending inputs from the segmental crawl oscillators were removed, the oscillatory membrane properties of R3b-1 were most likely a direct consequence of the DA application.

Matching the neuronal architecture of cell R3b-1 with its role as a bilateral and intersegmental coordinating interneuron

R3b-1 has been described as a decussating descending projection interneuron with a soma is located in the second sub-packet of the third neuromere (R3b) of the subesophageal ganglion (SEG) (Esch et al., 2002); the cells are bilaterally paired. Through a series of physiological and anatomical studies, the axon of R3b-1 has been shown to descend uninterrupted throughout the multiple ganglia comprising the nerve cord (schematic Fig. 4-2A; Esch et al., 2002). R3b-1 has a characteristic morphology within the SEG that clearly differentiates it from other projection neurons located within the R3b packet (Esch et al., 2002; Mesce et al., 2008). Arborizations extend from the primary neurite into neighboring sub-packets (R2b, R3b, R4a and R4b) and are typically restricted to their medial half (Fig. 4-2B).

The morphology described above, however, does not explain how a single neuron has the ability to coordinate both left and right halves of the body during crawling, which has left-right symmetry of body movements. At the level of the segmental ganglia, this issue was resolved. We observed fine punctate branches emanating from the unilateral axon and some of these terminal processes crossed the midline to invade the contralateral hemi-ganglion. In Figures 4-1C and 4-1D punctate decussating processes are shown in two different segmental ganglia. These anatomical data support the idea that a singular R3b-1 interneuron can communicate with crawl oscillatory networks residing in both hemispheres.

Activity from a single R3b-1 is necessary and sufficient for the expression of coordinated fictive crawling in whole nerve cord preparations

The anatomical data above support the premise that R3b-1 can communicate with crawl oscillatory networks residing in both hemispheres. To date, however, the possibility remains that bilateral motor coordination stems from some type of coupling via the contralateral homologue (Fan et al., 2005) or via an intervening coupling neuron (Muller and Scott, 1980). To address this potential, we transected the hemi-connective housing the R3b-1 homologue, for example, we transected the left hemi-connective below M1 to remove the R3b-1 located on the right of the ganglion (soma) (see Fig. 4-3A, micrograph). Both the median Faivre's nerve and contralateral hemi-connective remained unaltered. Because only a single R3b-1 cell remained, this manipulation also enabled us to test whether R3b-1 was both necessary and sufficient for crawling behavior.

Figure 4-3B shows the results of the above protocol in combination with 100 μ M DA added to the preparation in an attempt to induce crawling (Puhl and Mesce, 2008). We observed slow crawl-like bursting among the unit oscillators, but crawl bursts were uncoordinated across the segments. In the example provided, the mean periods for crawl-like activity were 26.89 ± 2.99 s, 28.92 ± 1.95 s and 26.35 ± 3.01 s for DP 8, DP 10 and DP 13, respectively. Importantly, when we injected +3 nA of current into the functionally remaining R3b-1, coordinated fictive crawling emerged (N=7). Intersegmental phase delays were 0.04 ± 0.008 between segments 8 and 10 and 0.04 ± 0.0007 between segments 10 and 13 (Fig. 4-3B). The periods of DE-3 bursting were shorter than those prior to R3b-1 stimulation and less variable between segments. For example, the periods during R3b-1 stimulation were 10.21 ± 0.65 s for DP8, 10.08 ± 0.71 s for DP10 and 10.24 ± 0.69 s for DP13. During current injection, we were unable to

observe R3b-1 spiking in the intracellular traces, therefore we determined when R3b-1 spikes occurred by analyzing the unit recordings in the hemi-connective (see methods). During current injection, R3b-1 spiking increased dramatically (Fig. 4-3B, raster across top). Prior to current injection (i.e., during uncoordinated crawl-like bursting), the peak frequency of R3b-1 spiking (during a 2 s window in the middle of the 3 bursts preceding current injection) was 8.5 Hz for the example shown (Fig. 4.3B). Three seconds after the onset of current injection, the R3b-1 spike rate was ca. 27 Hz, which induced coordinated fictive crawling. Increases (i.e., > 75%) in the rate of R3b-1 spiking during the genesis of coordination, was observed in all preparations analyzed (N = 4). These results indicate that spiking activity in a single R3b-1 neuron is sufficient to provide the intersegmental coordination needed to produce all the defining features of crawling behavior (Puhl and Mesce, 2008; Puhl and Mesce, 2010).

Next, we tested whether cell R3b-1 was necessary for the expression of coordinated fictive crawling. We again severed a single hemi-connective ipsilateral to the recorded R3b-1 soma to eliminate the influence of the contralateral homologue. We induced coordinated fictive crawling using 100 μ M DA (Puhl and Mesce, 2008) and injected -4 nA of current to reduce R3b-1 spiking, which we verified using the connective recording (Fig. 4-3C, top raster trace). Immediately after reducing R3b-1 spiking, we observed a detrimental interruption in the periodicity of DE-3 bursting and, most telling, a change in the coordination of DE-3 bursting across all 3 DP nerve recordings (N=5).

To confirm that a single R3b-1 was sufficient for crawling and to control for the possibility that R3b-1 might not activate a contralateral descending homologue located in a posterior segmental ganglion, we performed dual ipsilateral hemi-connective transections anterior and posterior to ganglion M2 (schematic; Fig. 4-4A). Prior to transection (day -1), sham controls (N=6) and experimental animals (N=6) exhibited

spontaneous crawling during the 8 minute observation period (Fig. 4-4B). After these transections, we observed overt crawling behaviors (Fig. 4-4B) in all experimental and sham control animals. Crawl periods for transected leeches before (day -1) and after (day +1) transection were 7.47 ± 2.17 s and 6.62 ± 0.83 s, respectively and were not significantly different ($P > 0.5$). Elongation durations on day -1 and day +1 were 4.55 ± 1.26 s and 3.75 ± 0.67 s, respectively and contraction durations were 1.41 ± 0.10 s and 2.11 ± 0.36 s on day -1 and day +1. The elongation and contraction durations were not significantly different after the transections compared to before ($P > 0.1$). Crawl movements from a single leech are shown in Fig. 4-4C, which provide additional confirmation that a single R3b-1 is sufficient for crawling.

Thus far, the behavioral attributes of R3b-1 fulfill the strictest criteria of a classical command neuron (Kupfermann and Weiss, 1978). As just shown, R3b-1 is both necessary and sufficient to activate crawling behavior, but how multifunctional can a command neuron be? Might this cell also be responsible for coordinating the individual crawl oscillators? If so, we predicted that it would be able to transiently reset the crawl rhythm by influencing the frequency of the crawl motor pattern. To test this idea, we drove R3b-1 with successively stronger current injections and measured whether the frequency of repeated crawl cycles increased. Again, we used 50-100 μ M DA to bias locomotor networks in the direction of crawling. Figure 4-5A shows that the frequency of R3b-1 spiking increased with larger amplitudes of injected current (N=5). The correlation coefficient for all data was 0.69. Establishing that R3b-1 spike frequency and current injection were, indeed, correlated we next measured the frequency of fictive crawling in response to elevated R3b-1 spiking. Figure 4-5B shows that the speed (frequency) of fictive crawling was faster when the frequency of R3b-1 spiking was higher (N = 5). The correlation coefficient for the dataset as a whole was 0.59. It is noteworthy that although

the frequency of fictive crawling increased (i.e., crawl cycles were shorter), the intersegmental phase relationships (across at least 3 DP recordings) were not significantly different in response to increased current injection (data not shown).

Another prediction is that spontaneous R3b-1 activity would exhibit bursting activity, which would be phase-locked to crawling. Furthermore, the peak of R3b-1 activity (spikes riding on slow waves) would be concurrent with the elongation phase of crawling because intracellular stimulation of R3b-1, *via* positive current injection, has been shown previously to stimulate the elongation of the nearly-intact body (Esch et al., 2002). To establish if our predictions were correct, we observed the activity of R3b-1 in the absence of any electrical stimulation during DA-induced fictive crawling. Figure 4-6 shows a representative example of the rhythmic oscillations observed in the membrane potential of R3b-1, which were phase-locked to fictive crawling (N=6). During each crawl elongation (i.e., times when DE-3 bursting is absent) R3b-1 spiking was at its peak (Fig. 4-6, gray parallelogram). During each crawl contraction, R3b-1 spiking was reduced as the cell became less depolarized (Fig. 4-6, dotted box). The phase relationships between the termination of R3b-1 bursts (Fig. 4-6, arrows) and DE-3 bursts (in DP4, DP7 and DP10) were 0.19 ± 0.006 , 0.33 ± 0.008 and 0.40 ± 0.012 , respectively, indicating that the bursting in each DP nerve occurred in succession from anterior to posterior and was phase-locked with the bursting of R3b-1.

Is R3b-1 synaptically coupled with motoneurons active during the elongation phase of crawling?

Based on the results above and on previous models of R3b-1 connectivity (Esch et al., 2002), we tested whether R3b-1 had a synaptic connection with the identified MN CV, a type of MN known to innervate the circular muscles active during the elongation

phase of crawling (Puhl and Mesce, 2008). Furthermore, we blocked local synaptic activity within and across 3 ganglia situated between the dual intracellular recordings of R3b-1 and MN CV (N=3) (schematic, Fig. 4-7A and gray box). This blockade did not interrupt fibers of passage. We reasoned that by blocking synaptic communication among the segmental crawl oscillators, any connections observed between R3b-1 and MN CV would be more direct (Puhl and Mesce, 2010; Tschuluun et al., 2001).

When R3b-1 was excited by intracellular current injection, we observed an increase in the frequency of CV spiking (Fig. 4-7B). Lower amplitude current injections led to a slight increase in CV spiking frequency (Fig. 4-7B, 1 nA), whereas stronger currents caused a marked increase in the frequency of CV spiking (Fig. 4-7B, 3 nA). Stimulation of any given single R3b-1 led to increased spiking in both the left and right CV MNs (data not shown). Figure 4-7C shows a portion of the R3b-1 and CV recordings over a finer timescale (denoted by gray lines). Although we did not see a 1:1 spike ratio in R3b-1 and CV, we did observe that for every R3b-1 spike produced an EPSP was present in the CV recording; in the example provided, the delay from R3b-1 spike onset to CV EPSP was 41.1 ± 0.29 ms (4.6 ms/segment). The delay for EPSPs in CV generated by an R3b-1 spike across all preparations tested was 5.17 ± 0.77 ms/segment (N = 4). To illustrate the relationship of R3b-1 spikes to CV EPSPs more clearly, the CV trace has been shifted to the left by ca. 41 ms and correlated activity has been highlighted by gray dotted lines (Fig. 4-7C). Across all preparations (N = 3), we observed an EPSP in the CV recordings for 98% of R3b-1 spikes (N = 100 spikes per preparation). In most cases, the EPSP not observed was replaced by a spike at the correct time. The relatively short delays, low variability in these delays, and synaptic blockade of 3 interposed ganglia strongly suggest that R3b-1 is directly exciting MN CV.

Fictive crawling requires both R3b-1 spiking and inter-oscillator drive

Puhl and Mesce (2010) demonstrated that descending cephalic signals are necessary and sufficient for the intersegmental coordination of fictive crawling, and it appears now that R3b-1 may well be the cellular correlate for this coordination. However, they also showed that local, inter-oscillator signals existed that can provide 'crawl-like' drive to neighboring oscillators. For any given segmental oscillator, this crawl-like drive extended primarily to the adjacent posterior two oscillators. Thus intersegmental coordination of the crawl oscillators was deemed to be a combination of long-distance projection signals from the cephalic ganglion and the local segmental crawl oscillators. If coordination were solely based on R3b-1's contributions, then intersegmental coordination will be preserved if communication among various crawl oscillators were blocked. To test this idea directly, we blocked local synaptic activity across 3 ganglia (Fig. 4-8A, schematic; gray box) while allowing R3b-1, a fiber of passage, to remain relatively unaltered (Fig. 4-7). We monitored fictive crawling (DP recordings) in ganglia anterior, within and posterior to the blocked region, and prior to, during and after the synaptic blockade. Intracellular recordings of R3b-1 were maintained throughout the experiments and DA (75-100 μ M) was used to bias locomotion towards crawling.

Prior to the synaptic blockade (Fig. 4-8B, Pre-Block, far left), coordinated fictive crawling was observed in DP4, DP7 and DP9. R3b-1 was phase-locked to the fictive crawling rhythm in a similar manner to what was described above (e.g., Fig.4-6). Upon blocking synaptic activity in ganglia M5-M7, fictive crawling was interrupted in those ganglia within and posterior to the blocked ganglia (Fig. 4-8B, Blocked, middle gray shaded region). Rhythmic bursting in R3b-1 was slightly degraded, although R3b-1 spiking continued. Fictive crawl-like bursting was relatively uninterrupted in DP4

(anterior to the block). In the block, rhythmic activity in DP7 was disrupted within 20 s of the synaptic blockade and all spiking activity was abolished within several minutes. In DP9 (below the block), rhythmic activity of DE-3 continued for several cycles and then ceased, although other smaller DP units were still observed. Shortly after removing the blockade, fictive crawl-like bursting continued in DP4 and coordinated bursting resumed in DP7 and DP9, with DP9 activity returning first (Fig. 4-8B, Post-Block, right). To eliminate the possibility that R3b-1 spiking frequency was simply not high enough to coordinate the oscillators during the block, we stimulated R3b-1 by injecting +3 nA of current during a different application of the synaptic blockade in the same preparation. Fig. 4-8C shows that even during stimulation of R3b-1, no activity was observed in the blocked ganglion (DP7) and that DE-3 bursting was absent in the ganglion posterior to the blocked ganglia (DP9). Similar to the un-stimulated condition (Fig. 4-8B), the ganglion anterior to the blocked ganglia (DP4) exhibited fictive crawl-like bursting of DE-3 prior to and during R3b-1 stimulation. Based on these data and that presented above, it is evident that cell R3b-1 is necessary and sufficient for crawling, but is only permissive for interoscillator coordination, which requires additional intersegmental drive.

Discussion

Overview

In this study we established that R3b-1 is critically important for the expression, maintenance and modulation of crawling. Specifically, R3b-1 activity is necessary for the expression and normal propagation of crawl-related bursting activity along the nerve cord (Fig. 4-3C). It is also sufficient for this bursting activity and thus normal fictive crawling (Fig. 4-3B). When considered in isolation, the results from Figure 4-3 categorize R3b-1 as a command neuron for crawling using the classical definition of

Kupfermann and Weiss (1978). Because our analysis moved beyond this initial assessment, the myriad contributions of R3b-1 for locomotion became increasingly apparent. Complementary to R3b-1's role in gating the expression of crawling, we found that the rate of R3b-1 spiking influences the speed (i.e., period) of ongoing fictive crawling by globally resetting the frequency of all of the segmental oscillators without interrupting their intersegmental coordination (Fig. 4-5). Thus R3b-1 activity also provides a means to control the speed of the entire crawl system from a centralized higher-order center. The above attributes render R3b-1 an exceptional cell, but its ability to coordinate the segmentally-distributed crawl oscillators makes this singular neuron quite noteworthy, and to our knowledge, provides the first biological example of a single command-like neuron that is essential for the intersegmental coordination of a locomotor behavior.

R3b-1 and the contribution of individual command-like neurons within a population

Many locomotion control systems are governed by populations of command-like neurons, however, individual cells within a given group may contribute differentially to specific outputs or modulations of behavior (Heinrich, 2002; Kristan et al., 2005; Dubuc et al., 2007; Jordan et al., 2008). For example, in the medicinal leech, a number of descending and ascending command-like neurons that control locomotion can be dedicated or be more multifunctional. The SE1 neuron promotes and the SIN1 inhibits swimming, and each cell appears to be dedicated to swimming (Brodfuehrer and Burns, 1995; Brodfuehrer et al., 1995). Cell E21, an ascending interneuron dedicated to swimming, can rapidly activate and prolong swim episodes and may be specialized for the activation of escape swimming (Mullins et al., 2010). Swim trigger neuron Tr-2 can initiate and at times terminate episodes of swimming (Brodfuehrer and Friesen, 1986;

O’Gara and Friesen, 1995). Although none of the swim-related command-like neurons discussed above are necessary for swimming (Brodfuehrer and Friesen, 1986; Puhl and Mesce, 2010), they provide a dynamic control mechanism over it.

In addition to its important role in crawling, cell R3b-1 can promote swimming behavior when sensory conditions are appropriate (Esch et al., 2002), thus this neuron has multiple functions across behaviors. Recently, Brodfuehrer et al. (2008) showed that Tr-1, a well-studied swim-trigger neuron, can also activate episodes of fictive crawling, although it is unclear whether such activation is *via* some interaction with R3b-1. Although R3b-1 is involved in multiple behaviors, its influence is most substantial for crawling where it is vital for locomotor initiation, coordination (Fig. 4-3) and frequency modulation (Fig. 4-5). Through the studies of identified projection neurons in the leech, a cohesive picture is emerging for how a diverse collection of cells can work together to select appropriate locomotor movements. For example, Tr-2 (as mentioned above) can terminate swimming; furthermore, this cell is electrically coupled to DA cells and DA is a potent inhibitor of swimming (Crisp and Mesce, 2004). Higher-order cells may also select accompanying whole-body postures needed for directed locomotor movements. Here again, the leech has provided insights into how posture-related descending cells may function in association with locomotor commands. In anatomical close proximity to R3b-1, two additional cephalic neurons, R3b-2 and R3b-3, have been shown to help shape specific body postures accompanying possible exploratory or turning motions overlaying crawling and swimming movements (Mesce et al., 2008).

Thus heterogeneity of function within cell populations and shared usage of command cells across behavioral routines appear to be a common theme emerging in the control of patterned behaviors. In vertebrates, such a control scheme emerges from studies of the lamprey (Deliagina et al., 2002). McClellan and Grillner (1984) determined

that microstimulation of many RS neurons can lead to the activation of fictive swimming, yet studies have also revealed that there is a heterogeneous distribution of functions within the RS population. For example, some RS neurons are dedicated to forward or backwards swimming, although the majority participates in both behaviors (Zelenin, 2011). Furthermore, some RS neurons active during swimming are also active during behaviors described as either backwards or forward crawling (Zelenin, 2005). An identifiable population of Müller RS neurons was shown to be insufficient to activate fictive swimming but some cells could induce bilateral changes in the frequency of ongoing (sensory-induced) fictive swimming while others produced ipsilateral excitation of ventral root bursts (motoneuron bursting) with a decrease in contralateral ventral root bursting (Buchanan and Cohen, 1982).

A variety of invertebrate animals also employ arrays of command-like neurons. Crustaceans utilize dedicated command-like fibers to either activate or terminate forward or backwards walking (Bowerman and Larimer, 1974) or to initiate swimmeret beating (i.e., swimming) (Weirsmas and Ikeda, 1964). Crickets possess command-like neurons that are devoted to a singular behavior but can impact those behaviors in multiple ways (i.e., trigger or inhibit, modulate speed, etc.) (Böhm and Schildberger, 1992; Hedwig, 2000). In the cockroach, stimulation of the command-like dorsal giant interneuron elicits running behavior, but the same cell can also elicit flight (Ritzmann et al., 1980). Although many of these results mirror those we obtained for R3b-1, we have yet to encounter a single cell that can activate two forms of locomotion and act as the intersegmental coordinating neuron for one of them, as can R3b-1.

The importance of context

Studies have provided evidence that higher-order neurons are sensitive to sensory context (Esch et al., 2002; Ritzmann et al., 1980) or the behavioral state (Nolen

and Hoy, 1984; Hedwig, 2000; Sasaki et al., 2006) of an animal. Our study highlights that a single neuron can have differential impacts on the selection, expression and timing of behaviors depending not only on sensory context but also the modulatory state of the system, establishing that R3b-1 is sensitive to two classes of context, sensory (the terrestrial/aquatic environment) and the modulatory milieu (DA). In the presence of DA, which biases the CNS away from swimming (Crisp and Mesce, 2004) and toward crawling (Puhl and Mesce, 2008), intracellular depolarization of R3b-1 can lock-in crawling and shift swimming to crawling in preparations lacking sensory input (Fig. 4-1A). In the absence of exogenous modulators, these events are not observed in isolated nerve cords (Esch et al., 2002). The ability of DA to modulate behavioral selection has been reported elsewhere as well. For example, in *Caenorhabditis elegans* DA signaling is associated with the mechanosensory encoding of water levels surrounding the body (Sulston et al., 1975), which determines the form of locomotion expressed (Pierce-Shimomura et al., 2008). The role of DA signaling in locomotor behavioral choice is further confirmed by preliminary evidence suggesting that DA is necessary and sufficient for the switch from swimming to crawling (Pierce-Shimomura et al., 2009; Mesce and Pierce-Shimomura, 2010). DA levels in this animal may also signify the presence of a high-quality food source, thus causing them to crawl more slowly (Sawin et al., 2000).

We also established that DA has effects on R3b-1 within the cephalic ganglion. For example, in cephalic ganglion preparations isolated from the rest of the nerve cord, R3b-1 generated DA-induced rhythmic oscillations having a crawl-like period (Fig. 4-1D). These oscillations resembled those observed during fictive crawling even when ascending information was blocked (Fig. 4-6). Thus DA can influence the decision to crawl at both levels of CNS organization: the segmentally-distributed crawl oscillators (Puhl and Mesce 2008) and higher-order command input (i.e., cephalic ganglion).

Studies of the amine modulation of swimming in the leech have also indicated dual anatomical targets of modulation, although serotonin was shown to have opposing effects on the nerve cord and cephalic ganglion (Crisp and Mesce, 2003; Crisp and Mesce, 2006).

R3b-1 and its connectivity

R3b-1 appears to play a particularly important role in the most flexible phase of crawling which is elongation (Figs. 4-6, 4-7; Stern-Tomlinson et al., 1986; Esch et al., 2002). In both nearly-intact (Esch et al., 2002) and isolated CNS preparations (Fig. 4-6), R3b-1 is most active (i.e., spike frequency) during the elongation phase of crawling. Our study provides the first report of a synaptic target for R3b-1, which we determined excites motoneurons involved in elongation (i.e., CV) (Fig. 4-7). This connection is likely a direct one because blockade of synaptic activity of interposed ganglia did not eliminate EPSPs observed in CV; furthermore, the variance of latency between R3b-1's action potentials and the EPSPs in CV was small.

Interestingly, fictive crawling can be initiated and maintained by rhythmic (Fig. 4-6) or tonic (Fig. 4-3) R3b-1 activity. Similarly, the command-like swim gating neuron (cell 204) in the leech can produce normal fictive swimming when it is stimulated to produce tonic impulses, however, under normal conditions its rhythmic activity patterns are phase-locked to the fictive swimming rhythm (Weeks and Kristan, 1978). One question that arises is whether or not the rhythmic activity of R3b-1 has any functional significance? We conclude that it does because of its crawl-like patterning that is independent of ascending inputs. The entire crawl motor system, however, is apparently flexible enough to maintain segmental rhythmicity in the absence of R3b-1 oscillations. Because the elongation and contraction phase kernels of the crawl circuitry are located

within each segmental “unit burst generator” (Puhl and Mesce, 2008), completion of both phases in a cycle can be the result of the initial descending gating of R3b-1, especially because R3b-1 is predominantly active during the elongation phase of crawling.

R3b-1 and a mechanism for the intersegmental coordination of crawling

Our results confirmed and extended a prediction we made previously that some type of descending higher-order signal(s) from the cephalic ganglion is combined with local (posterior-directed) inter-oscillator coupling to achieve the intersegmental coordination observed during crawling (Puhl and Mesce, 2010). Based on our current results, we postulate that a single R3b-1 neuron makes synaptic connections with each of the segmental crawl oscillators; our anatomical data support this idea (Fig. 4-2). In each segment, these inputs together with local oscillator-to-oscillator coupling combine as a logical AND gate to ensure the sequential activation of the various crawl oscillators (schematic, Fig. 4-9). For any given segmental crawl oscillator, local information from the adjacent anterior oscillator or from R3b-1 will be sub-threshold for activating the segmental rhythm-generators for crawling. Our data support this conclusion because when we blocked the inter-oscillator coupling among three interposed ganglia (high Mg^{++} , low Ca^{++}), the loss of oscillator input prevented the adjacent untreated (posterior) ganglion from bursting in a crawl mode (Fig. 4-8). We controlled for the potential loss of spiking activity in R3b-1 under this type of synaptic blockade by demonstrating that a synaptic target of R3b-1 (MN CV) continued to receive a 1:1 spike to EPSP ratio (Fig. 4-7C). When the converse experiment was conducted, whereby R3b-1’s input was silenced with hyperpolarization (the contralateral R3b-1 was also functionally removed), the intersegmental coordination was lost, although crawl-like bursting was evident due to previous DA activation (Fig. 4-3C). Coordination was once again restored when we

electrically stimulated R3b-1 (Fig. 4-3B). Thus only when appropriate targets receive dual signals simultaneously can individual crawl oscillators progressively become activated, forming a metachronal wave of posteriorly-directed crawl activity. When all the independent crawl oscillators are activated by DA, we have found that only R3b-1 intracellular activity is sufficient to coordinate them. Our data support the idea that R3b-1 not only serves as a CNS-wide global activation signal, but *permits* the concatenated posterior-directed inter-oscillator signals to work in parallel. Our data also show that a threshold of R3b-1 spiking is likely needed to permit intersegmental coordination. In some preparations, DA-induced crawl-like activity did not become coordinated across segments until R3b-1 spiking was sufficiently elevated through intracellular current injection (Fig. 4-3B). Because R3b-1 spike rates vary between individuals, this threshold is not absolute but rather some relative increase in rate (e.g., >75%) as we observed.

Our working model also accounts for the flexible nature of crawling. For example, crawling behavior in intact leeches can be paused mid-cycle with other behaviors expressed during a pause (Stern-Tomlinson et al., 1986). If a decision is made such that R3b-1 activity is suppressed, one of the inputs to the segmental crawl oscillator AND gates will be lost and propagation of the command to crawl may be terminated throughout the entire CNS (Fig. 4-9).

Our model for intersegmental coordination offers an explanation for how relatively independent oscillators can be phase-coupled by a combination of local and long-distance signals. A model with similar core features was proposed to describe a potential mechanism for lamprey swimming, which employed a chain of non-linear oscillators coupled locally and by longer-distance projecting fibers (Cohen and Kiemel, 1993). Despite differences in some of the anatomical details, our study reflects many predictions made in the lamprey model.

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Figures

Figure 4-1: DA biases R3b-1 intracellular stimulation to induce fictive crawling (**A**) and induces rhythmic oscillations in R3b-1 with a period similar to fictive crawl cycles (**B-D**).

A, Number of isolated nerve cords (N=24) in which R3b-1 was injected with positive current (+3-4 nA) and fictive locomotion was monitored. Prior to the superfusion of 75-100 μ M DA, 5/24 nerve cords exhibited fictive swimming, and no switching between fictive swimming and crawling was observed. After DA superfusion, 100% of preparations exhibited fictive crawling exclusively. **B**, Schematic of the isolated cephalic ganglion preparation and placement of electrode to record R3b-1 (see Methods for cell identification). **C**, Intracellular R3b-1 recording prior to application of DA; note lack of bursting activity. **D**, Intracellular recording from the same R3b-1 neuron shown in (**C**) after a treatment with 100 μ M DA. All scalebars in (**C**) also apply to (**D**).

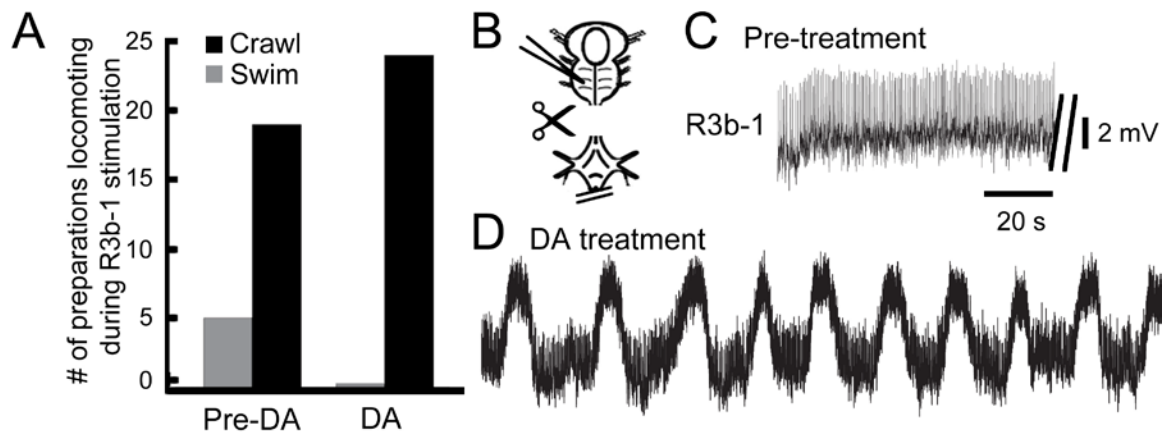


Figure 4-1: DA biases R3b-1 intracellular stimulation to induce fictive crawling and induces rhythmic oscillations in R3b-1.

Figure 4-2: Morphology and projection pattern of the cephalic-originating neuron R3b-1.

A, Schematic showing the location of R3b-1's soma and decussating primary neurite.

The axon of this cell projects uninterrupted throughout the nerve cord; note only one of

the R3b-1 pair is shown. **B**, Laser scanning confocal micrograph of R3b-1 [dorsally

situated within the subesophageal ganglion (SEG)] that has been ionophoretically

injected with Neurobiotin and visualized with Cy3 fluorescence. The neuromeres are

labeled. **C**, Axon of R3b-1 projecting through M1. The majority of punctate arborizations

ramify throughout the ipsilateral side of the ganglion, however, several arbors invade the

contralateral half of the ganglion (arrows). **D**, Second example of R3b-1's axon within

M1 but at a higher magnification (inset shows the entire ganglion). Arrows identify a

terminal branch that crosses the midline. All scale bars are 100 μm .

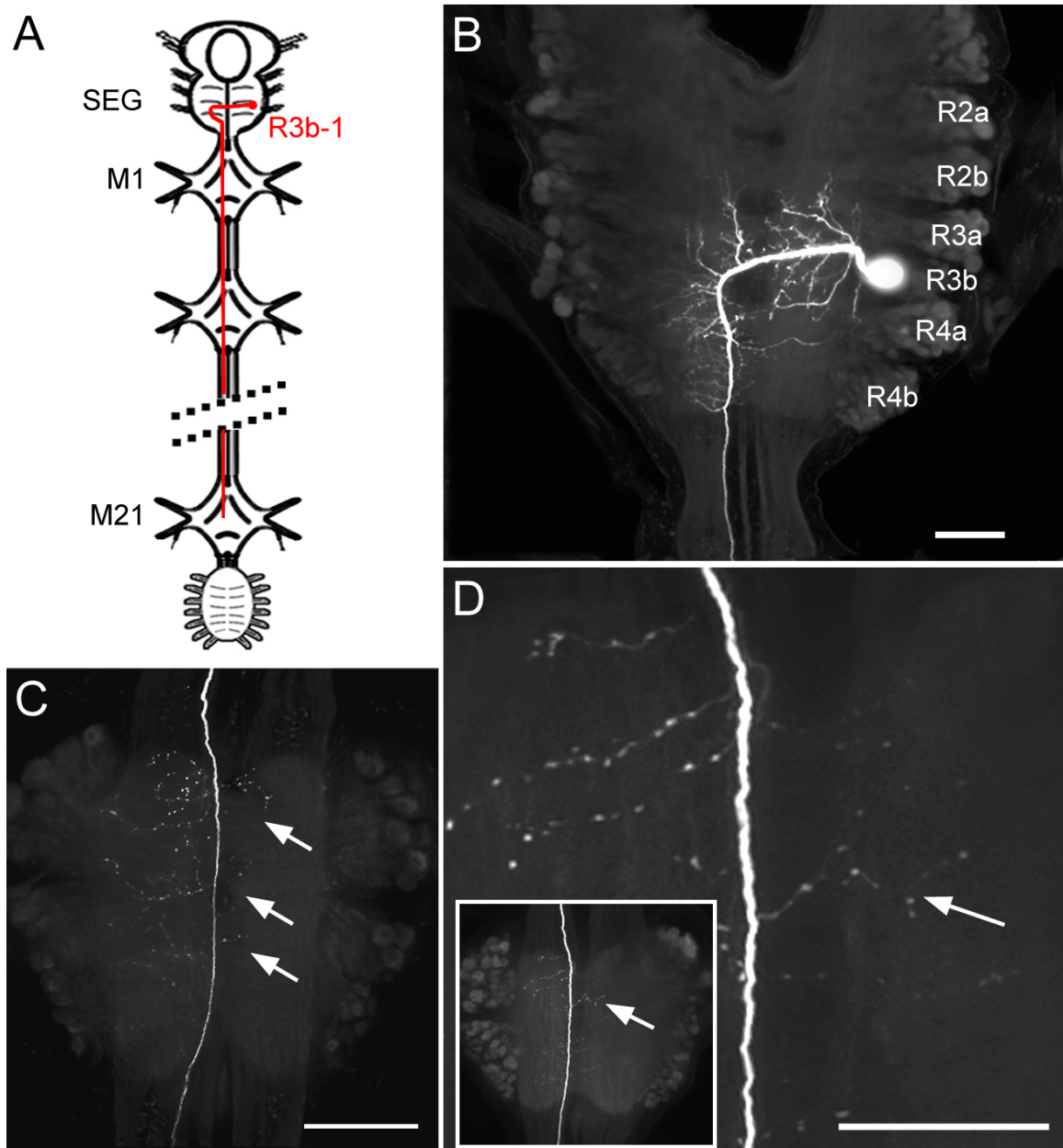


Figure 4-2: Morphology and projection pattern of the cephalic-originating neuron R3b-1.

Figure 4-3: Spiking activity in R3b-1 is necessary and sufficient for coordinated fictive crawling. **A**, Confocal micrograph (left) of a transected hemi-connective between M1 and M2, which disrupted information flow along the axon of the contralateral R3b-1 homologue (stylized neuron). The R3b-1 from which we recorded was filled with Neurobiotin. Note that Faivre's nerve and the contralateral hemi-connective were not damaged. Schematic (right) depicts recording sites in the cephalic ganglion and DP nerves. **B**, Intracellular positive current injection into R3b-1 (+3 nA; second trace) is sufficient to convert DA-induced uncoordinated crawl-like activity into coordinated fictive crawling. Top trace is a raster of R3b-1 spike times recorded in the connectives between M2 and M3. Fictive crawling was monitored in 3 dorsal posterior (DP) nerves (bottom 3 traces). The largest unit in the DP traces belongs to the dorsal longitudinal motoneuron DE-3 which is active during the contraction phase of fictive crawling (Puhl and Mesce, 2008). Gray bar denotes +3 nA current injection into R3b-1 (intracellular trace is off-scale). The peak rate of R3b-1 spiking prior to and during current injection was 8.5 Hz and 27 Hz, respectively (data not shown). **C**, Negative current injection into R3b-1 interrupted the intersegmental coordination that defines fictive crawling (Puhl and Mesce, 2010). Collection of cells recorded is the same as in (**B**). Gray bar denotes time of -4 nA intracellular current injection (intracellular trace is off-scale). Recordings in **B-C** were obtained in preparations treated with 100 μ M DA, which was washed off shortly prior to the recordings.

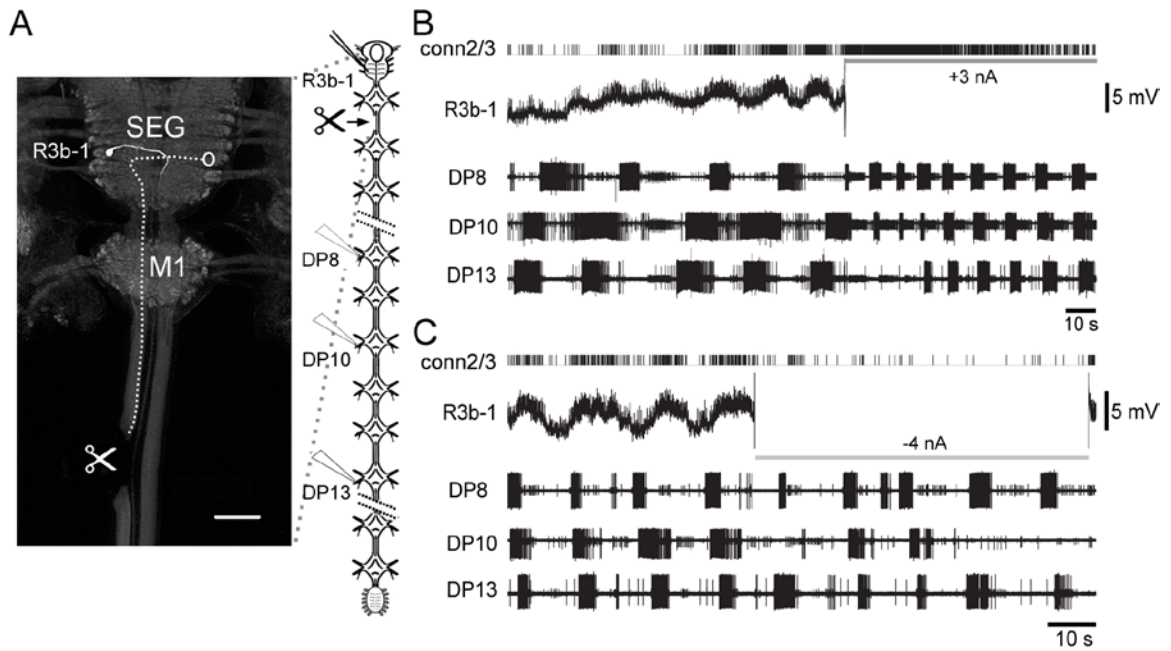


Figure 4-3: Spiking activity in R3b-1 is necessary and sufficient for coordinated fictive crawling.

Figure 4-4: Transection of ipsilateral hemi-connectives anterior and posterior to a segmental ganglion eliminate descending information from one of the pair of R3b-1 neurons, but does not interrupt crawling behavior in otherwise intact leeches. **A**, Schematic illustrating the location of the dual ipsilateral hemi-connective transections. **B**, The number of spontaneous crawl cycles observed in 8 minutes was not significantly different in transected leeches (N=6) and sham control leeches (i.e., received surgery but no transection) (N=6). **C**, Crawling behaviors observed one day prior to and one day after dual ipsilateral hemi-connective transection were qualitatively similar. After transection, the duration of the movements was slightly longer, however, crawling behaviors were otherwise indistinguishable from those observed in sham control leeches (data not shown).

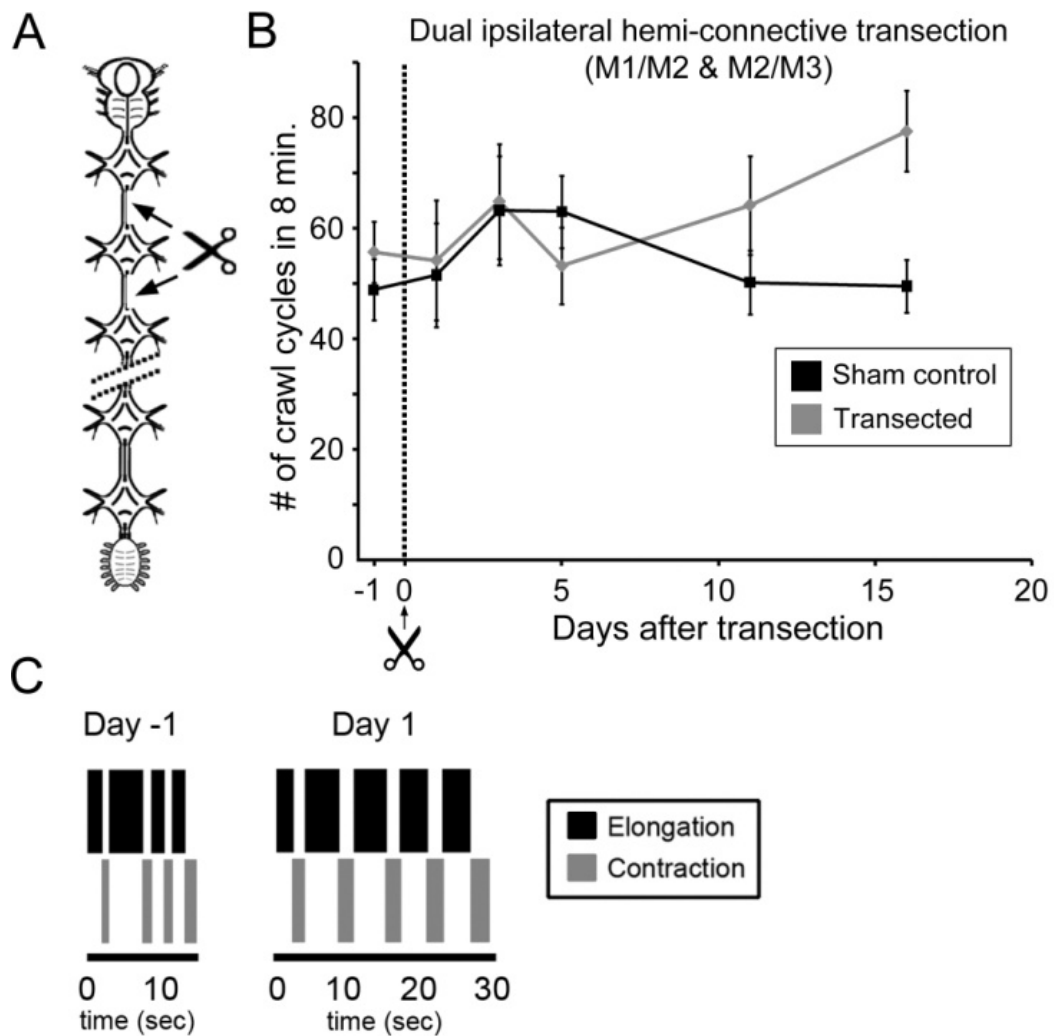


Figure 4-4: Transection of ipsilateral hemi-connectives anterior and posterior to a segmental ganglion and its effects on crawling.

Figure 4-5: Scatter plots showing that the frequency of R3b-1 spiking modulates the speed of fictive crawling. **A**, Graph showing that with increased stimulus amplitude the frequency of R3b-1 spiking also increases. **B**, Graph showing that the rate of R3b-1 spiking is positively correlated with the frequency of fictive crawling. Data depicted in both graphs were obtained from the same preparations (N=5).

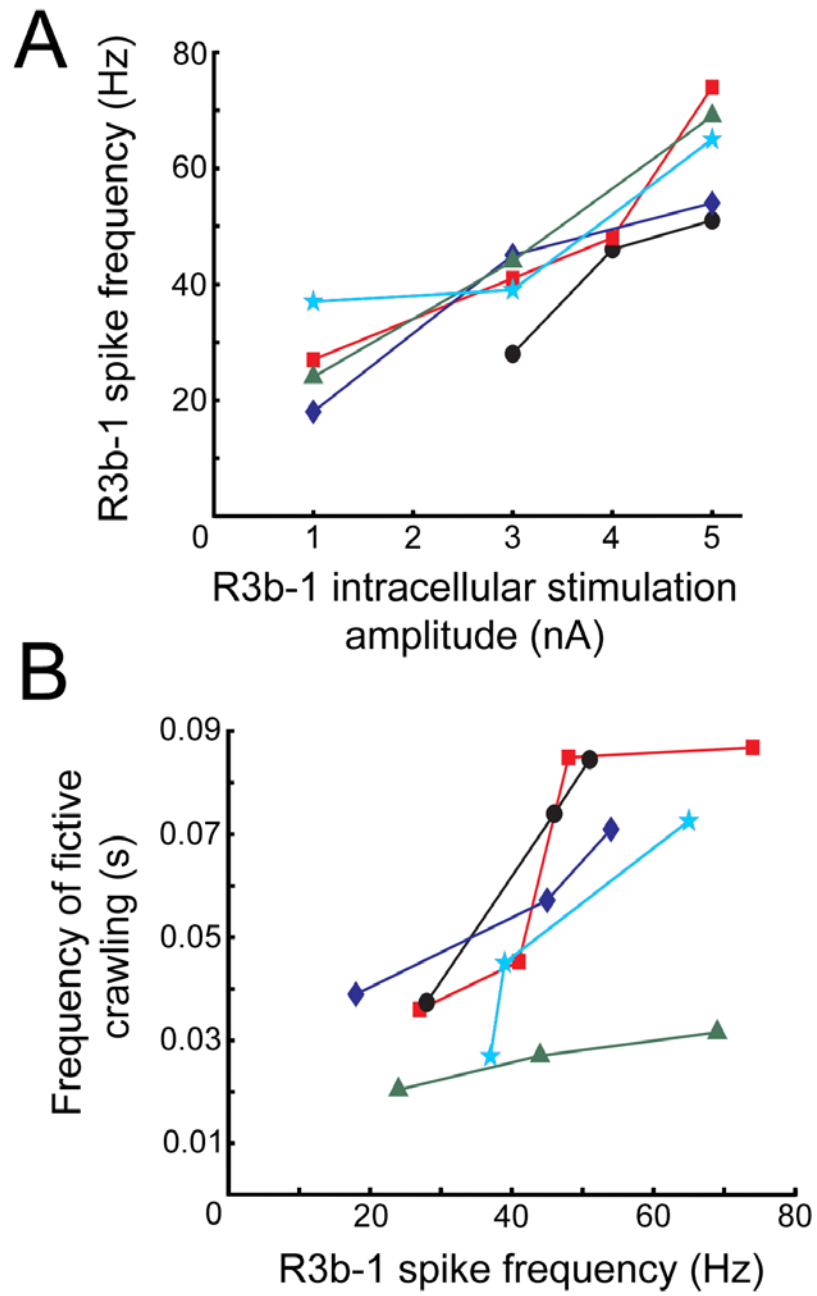


Figure 4-5: The frequency of R3b-1 spiking modulates the speed of fictive crawling.

Figure 4-6: DA-induced spontaneous (i.e., no current injection) bursting in R3b-1. Motoneuron (MN) bursting during both the elongation (gray shading) and contraction (dotted box) phases of each fictive crawl cycle are phase-locked with R3b-1 rhythmic activity. Note that the metachronal wave of DP activity propagates along the nerve cord in an anterior to posterior direction. Arrows denote both the times when the depolarized state of R3b-1 is sharply reduced and the transition points from elongation to contraction.

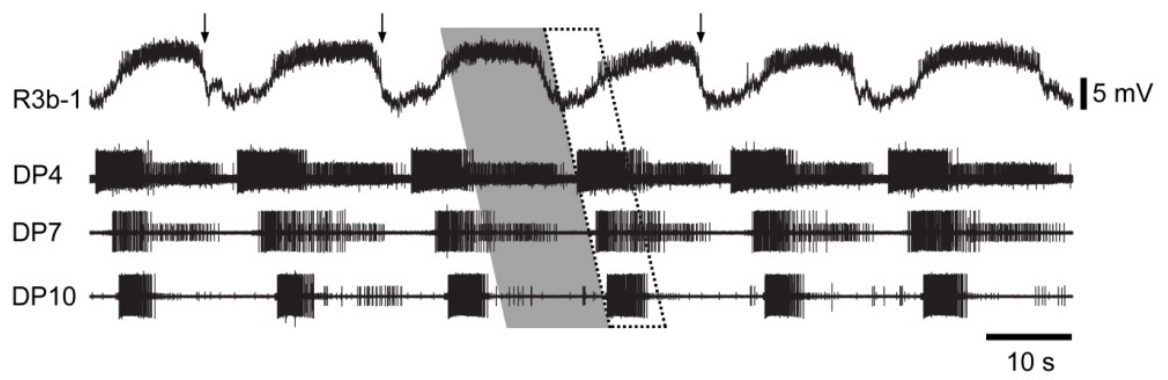


Figure 4-6: DA-biased spontaneous fictive crawling is phase-locked to R3b-1 bursting.

Figure 4-7: The crawl-related MN CV is a synaptic follower of R3b-1, likely *via* a direct route independent of inter-ganglionic circuits. **A**, Schematic showing electrode placement of intracellular recordings and location of synaptic blockade across 3 segmental ganglia (gray shaded box). This blockade disrupted inter-oscillator coupling (M5-M7) without perturbing information flow along the fibers of passage (i.e., R3b-1). **B**, Positive current injections (+1nA and +3 nA) led to excitation of the CV MN in M9. **C**, Expanded timescale of the data bracketed by vertical gray bars in **(B)**. Electrophysiological traces obtained from the CV MN (in M9) have been shifted to the left by 41 ms to show more clearly the relationship of R3b-1 spikes and EPSPs observed in CV. Dotted gray lines connect R3b-1 spikes to their corresponding CV EPSPs. Under similar conditions we observed CV EPSPs in response to R3b-1 stimulation (N=4).

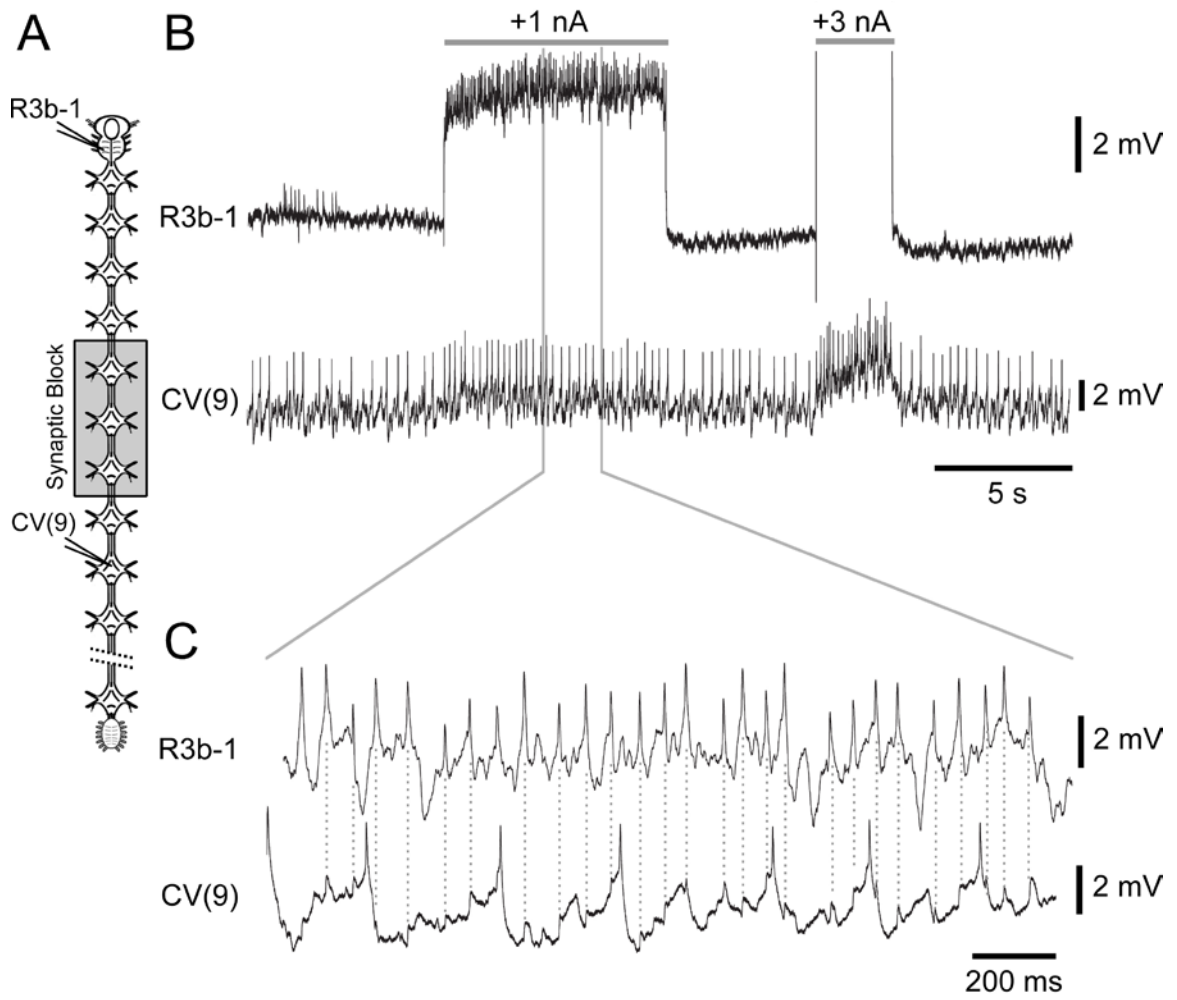


Figure 4-7: The crawl-related MN CV is a synaptic follower of R3b-1.

Figure 4-8: Fictive crawling requires parallel inputs from R3b-1 and local inter-oscillator circuits. **A**, Schematic showing sites of intracellular and extracellular recordings and the blockade of inter-oscillator signaling (gray shaded box). **B**, During blockade of inter-oscillator coupling, fictive crawling within (gray shading) and below (dotted gray box) the blocked ganglia was interrupted. Intracellular R3b-1 recording (top trace) and DP recordings from M4, M7 and M9 before (Pre-Block; left), during (Blocked; gray shaded region) and after (Post-Block; right) blockade of synaptic activity by treatment of M5-M7 with high-Mg⁺⁺/Low-Ca⁺⁺ saline. Note that intersegmental coordination was present before and after blockade of synaptic activity using High Mg⁺⁺/Low Ca⁺⁺ saline (dotted black lines). **C**, Recording from the same preparation presented in **(A)** showing the effects on fictive motor outputs of injecting +3 nA into R3b-1 (gray bar above trace) during synaptic blockade of M5-M7. Note: smaller unit in DP9 recording in **(C)** is not DE-3. Horizontal scalebar in **(B)** also applies to **(C)**. The entire preparation was treated with 100 μ M DA.

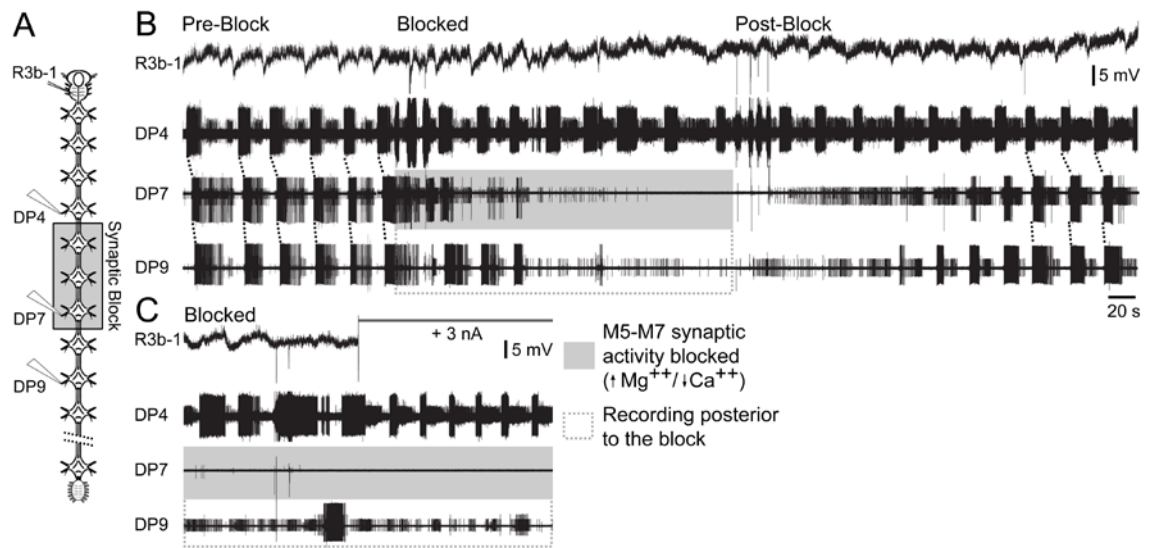


Figure 4-8: Fictive crawling requires parallel inputs from R3b-1 and local inter-oscillator circuits.

Figure 4-9: Schematic depicting the AND-gate model underlying the intersegmental coordination of crawling behavior in the medicinal leech. The inputs to the AND gate are from R3b-1 (thinner black arrows) and the anterior adjacent crawl oscillator (thicker black arrows). During crawling, R3b-1 signals are continuously present throughout the elongation phase. This R3b-1 excitation is summed with the segmental oscillator drive extending from the anterior adjacent oscillator. When the conditions of the AND gate are met, successively posterior crawl oscillators will become activated with appropriate phase relationships. Because each ganglion contains a unit burst generator for both the elongation and contraction phases of a crawl step, once the signal for crawl activation is gated, the full two-phase output will be generated (assuming a built-in delay for contraction onset).

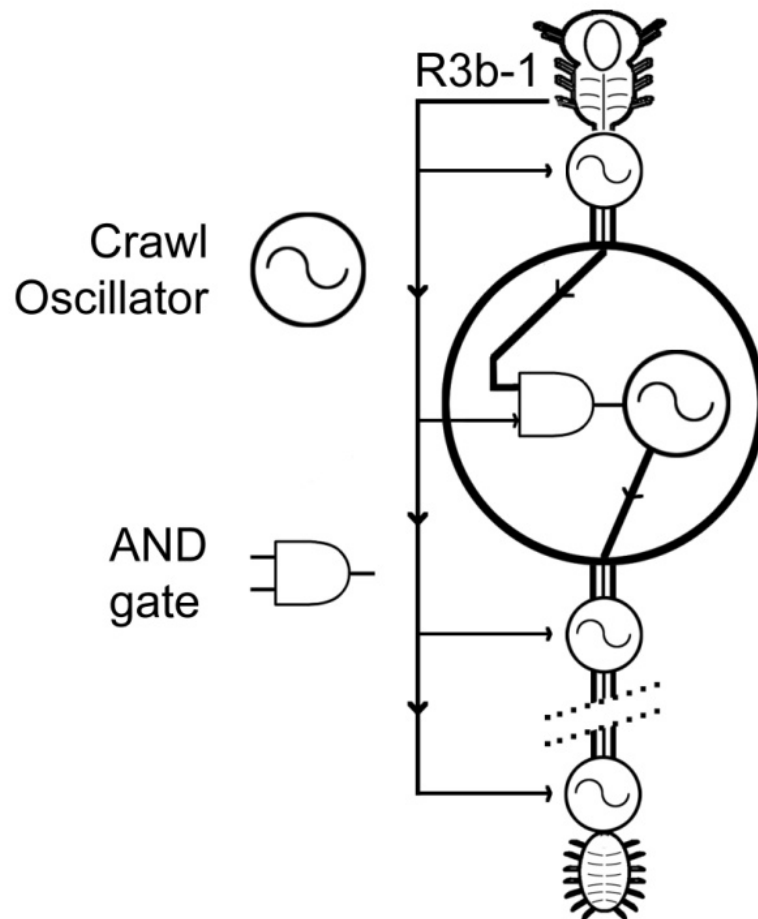


Figure 4-9: Schematic depicting the AND-gate model underlying the intersegmental coordination of crawling behavior.

Chapter Five:

**From intact animal to individual neuron: Studying the neural correlates of
a flexible locomotor behavior at the level of networks and cells**

My thesis research has focused on the neural mechanisms that underlie a flexible locomotor behavior i.e., crawling. My work has investigated these mechanisms at the levels of the neural network and individual neuron. Crawling in the medicinal leech (*Hirudo verbena*) was studied because it possesses great flexibility in its movements but is simpler than walking, especially in vertebrates (Stern-Tomlinson et al., 1986). The medicinal leech has been an almost ideal model for my studies because it expresses two easily discernable forms of locomotion, is metamerically segmented, and has only about 10,000 neurons in its entire CNS, many of which are uniquely identifiable between individuals (see Chapter 1). These features have aided me in understanding the neural bases of crawling, as well as locomotor behaviors, behavioral switching and the higher-order control of behaviors in general.

A behavioral role for dopamine

The biogenic amine dopamine (DA) has been implicated in controlling a wide range of animal behaviors throughout the evolutionary spectrum. Surprisingly, few examples have documented a specific behavioral role for DA in any animal. Indirect evidence has suggested that DA plays some role in leech biting behavior (O’Gara et al., 1991). More recently, Crisp and Mesce (2004) provided the first demonstration that DA, when bath applied to isolated nerve cords, terminates fictive swimming. In Chapter 2 of my dissertation, I provide the first evidence that, in addition to turning swimming off, DA promotes crawling in intact but isolated CNS preparations. I verified that rhythmic activity induced by DA represented authentic (fictive) crawling by establishing that key crawl-related motoneurons were active as they are during overt crawling, and I established that the anterior-to-posterior metachronal wave of crawl bursting matched overt crawling (Eisenhart et al., 2000). I also showed that DA activates the full

complement of both excitatory and inhibitory MNs of crawling. My results provide the first example in any animal of DA being sufficient to activate a complete, robust and sustained motor program for locomotion in a single isolated division of the CNS, i.e., a single ganglion. Furthermore, the ability to turn off swimming and turn on crawling has established that DA may act as a chemical decision maker in the CNS. This idea is further supported by the fact that some of the DA-synthesizing neurons located within the cephalic ganglion (Crisp et al., 2002) are dye-coupled (i.e., electrically coupled) to a command-like neuron, Tr-2, that can terminate swimming (Crisp and Mesce, 2004).

Location and extent of the central pattern generator for crawling

The ability of DA to activate the full motor program for crawling in a single ganglion gave me an important insight into the architecture of the neural networks that control crawling. Because DA activated both excitatory and inhibitory motor outputs, and both phases of the crawl cycle, I established that a complete set of rhythm-generating elements (i.e., a CPG or unit oscillator) is housed within a single ganglion. In Chapter 2, I also determined that ganglia from all regions of the CNS produce fictive crawling in response to DA. In fact, during the course of my thesis research, I have observed DA-induced fictive crawling in every segmental ganglion from M2 to M20. Thus, I elucidated that the crawl CPGs are homogeneously distributed along the nerve cord, creating a chain of phase-coupled yet relatively independent neural oscillators that can generate crawling movements, although assistance from descending inputs is necessary.

In contrast to crawling, the CPGs for swimming are unevenly distributed throughout the CNS. When fictive swim-inducing stimuli are delivered, isolated ganglia from the middle-body region are best able to produce fictive swimming (Hocker et al., 2000). Isolated ganglia from the anterior body region are able to produce fictive

swimming, but with less efficacy and the resulting bursting is not as robust and sustainable; ganglia from the posterior body region are unable to exhibit fictive swimming in isolation. Although all ganglia produce the appropriate motoneuron bursting during fictive swimming in the intact CNS, the rhythm-generating circuitry that generates these bursts are clearly more heterogeneously distributed than those for crawling (Hocker et al., 2000).

Mechanisms of intersegmental coordination for a flexible locomotor behavior

A key set of results from my thesis research has elucidated some of the mechanisms underlying the coordination of the relatively independent CPGs for crawling, and Chapter 3 has built upon Chapter 2. A fundamental “problem” that the leech CNS must solve is to coordinate the relatively independent segmental crawl CPGs to produce the anterior-to-posterior metachronal wave of motor activity observed in the intact animal and in the isolated but whole CNS (i.e., fictive) preparation. I began Chapter 3 by providing data showing that descending signals from the cephalic ganglion (i.e., the brain) are both necessary and sufficient for overt and fictive crawling and the intersegmental coordination of the crawl CPGs. These results also established that sensory information is not sufficient to drive and coordinate the segmental crawl CPGs as it can for swimming behavior. These results directly contrast with those for leech swimming. For example, Brodfuehrer and Friesen (1986) established that signals descending from the cephalic ganglion are not necessary for the expression of coordinated fictive swimming. My work on swimming behavior in nearly-intact leeches in Chapter 3 confirms these results.

I also determined that the individual crawl CPGs transmit crawl-like excitatory drive to adjacent crawl CPGs (Chapter 3). To do this I took advantage of DA’s ability to

activate the crawl oscillators directly. Upon activating fictive crawling in one ganglion only, in short (4-6) chains of segmental ganglia lacking connections to the cephalic ganglion, I observed robust rudiments of fictive crawling in the untreated posterior adjacent ganglion. I observed similar but significantly weaker crawl-like bursting in ganglia located two segments away, and in the anterior adjacent ganglion. Importantly, normal intersegmental phase delays were absent in these chain preparations. These results confirmed further the necessity of cephalic signaling for normal intersegmental coordination of the crawl CPGs. These data also established that the strength of segmental communication is in the posterior direction. The results from Chapter 3 led to the hypothesis that a combination of descending cephalic information and local inter-CPG signals work together to produce the posterior-directed metachronal wave of crawling movements. I tested this hypothesis in Chapter 4 (see below).

The results from Chapters 2 and 3 provided me with essential insights into the underlying systems/network-level architecture of the neural networks that control crawling behavior. In Chapter 4, I aimed to identify individual neurons that comprise the descending cephalic coordination signals. Because the crawl CPGs are relatively independent and are reliant upon long-distance cephalic signals, I was in a unique position to reveal a novel mechanism for the inter-oscillator coordination of a locomotor behavior.

I focused my studies in Chapter 4 on a cephalic interneuron that projects along the entire CNS, which is named R3b-1 (Esch et al., 2002). R3b-1 was previously found to activate crawling or swimming in nearly-intact leeches based on the fluid levels surrounding the body (Esch et al., 2002). It also activates fictive crawling or swimming in isolated nerve cords, although the specific form is less predictable. I chose to study this

cell because it had the potential to communicate with all the segmental crawl CPGs and was already implicated in activating crawling.

In Chapter 4, I determined that R3b-1 is both necessary and sufficient for coordinated fictive crawling in isolated nerve cord preparations. This result establishes that R3b-1 serves as a command or command-like neuron for crawling behavior based on the classical definition proposed by Kupfermann and Weiss (1978). Additionally, R3b-1 modulates the speed of fictive crawling. In addition, R3b-1 fires rhythmic bursts of activity that are in-phase with the elongation phase of the crawl rhythm, and it communicates directly with the CV motoneurons in the segmental ganglia that give rise to elongation movements. I further confirmed the relationship of R3b-1 and CV using voltage-sensitive dye (VSD) imaging techniques developed to identify synaptic targets of a given neuron (Fig. 5-1) (Cacciatore et al., 1999). Briefly, I injected 3 nA, 1 Hz current pulses into R3b-1 in an isolated whole CNS preparation. I loaded the VSDs into cells in M10 and imaged their optical activities looking for optical signals that were coherent (i.e., phase-locked) to the R3b-1 recording. I identified that both CV motoneurons were coherent with R3b-1 as well as several other unidentified neurons on the ventral surface of M10. To date, my results provide the only known synaptic targets of R3b-1. Taken together, these results show that a single 'command-like' neuron can have a multi-dimensional influence over a given behavior. These data also provide the first biological example of a 'coordinating' command-like neuron for a flexible multi-CPG behavior.

That R3b-1 is necessary for the intersegmental coordination of the segmental crawl CPGs is an exciting and novel result. A question that remained, however, was whether R3b-1 was solely responsible for creating the relatively slow (ca. 1 s/segment) metachronal wave of CPG activation observed during crawling. As I stated above, I hypothesized that in addition to the descending cephalic signals (i.e., R3b-1 spiking), the

local concatenated inter-CPG signals (elucidated in Chapter 3) were also necessary for appropriate intersegmental coordination. To test this hypothesis, I interrupted the CPG-to-CPG concatenated signaling by blocking local synaptic activity across several consecutive segmental ganglia using a high-Mg⁺⁺/Low Ca⁺⁺ saline (Chapter 4). Importantly, I established that this blockade did not interfere with R3b-1 signaling. Blocking the concatenated inter-CPG signals, however, did interrupt the propagation of fictive crawling, indicating that these signals are also required for the expression of fictive crawling.

Based on these results I posit that when the CNS enters into a crawl mode the inputs to a given segmental crawl CPG function as a logical AND-gate to ensure that each CPG is activated only if it receives simultaneous signals from the cephalic ganglion *via* R3b-1 and the adjacent anterior CPG (Chapter 3). Although R3b-1 is a necessary component for intersegmental coordination of the crawl CPGs, R3b-1 does not necessarily provide the specific timing signals for sequential CPG activation but rather **permits** the concatenated inter-CPG signals to activate the segmental oscillators sequentially. The timing is likely provided by the concatenated inter-CPG signals themselves. At times when R3b-1 is bursting in 'crawl-time', the crawl rhythm is likely to be especially stereotypic and robust.

Higher-order command-like neurons can have multi-dimensional effects on behaviors

In Chapter 4, I aimed to demonstrate the influence that R3b-1 can have on a leech's behavior. What I learned was that even a single command-like neuron can influence multiple aspects of a single behavior, and can contribute to the generation of multiple behaviors. In the previous section, I described the multiple contributions that R3b-1 has for crawling behavior that I elucidated in Chapter 4. Now, I want to shift

themes to note that R3b-1 also serves as part of a decision-making system to select the appropriate form of locomotion within a given context (Esch et al., 2002; Chapter 4).

In addition to the sensory context of the body, I determined in Chapter 4 that the form of motor outputs generated by R3b-1 is also susceptible to modulation by DA. In isolated nerve cords and in the absence of exogenous modulators, R3b-1 activates either fictive swimming or crawling. In any given preparation, the form of fictive locomotion induced by R3b-1 electrical stimulation remains unchanged (i.e., if R3b-1 activated fictive swimming the first time it would do so for all subsequent episodes). However, after applying DA to isolated nerve cords that first produced fictive swimming in response to R3b-1 stimulation, 100% of preparations switched and exhibited fictive crawling. Furthermore, R3b-1 exhibited rhythmic oscillations that resembled fictive crawling when I treated cephalic-ganglion preparations with DA (note: ganglia were not connected to the rest of the CNS). These results provide evidence that DA biases the CNS both at the levels of the segmental ganglia (Chapter 2) and the cephalic ganglion (Chapter 4) to exhibit crawling. In the context of swimming in the leech, the cephalic ganglion and the segmental swim CPGs are both susceptible to modulation by serotonin, however, serotonin inhibits swimming when applied to the cephalic ganglion (Crisp and Mesce, 2003) and promotes it when applied to the segmental ganglion (Willard, 1981).

Thus my results, along with those from Esch et al. (2002), establish that R3b-1 has multi-dimensional contributions to the selection and generation of locomotor behaviors in the medicinal leech. My work provides one of only a few examples of a single neuron that participates in multiple aspects of multiple behaviors and is the only higher-order 'coordinating' neuron described to date.

Future Studies and Preliminary Data

My dissertation research and the work of others who have studied crawling in the medicinal leech (reviewed: Kristan et al., 2005) have established a solid foundation for understanding the neural mechanisms of crawling. Of course many details remain unknown. For example, sensory information from mechanoreceptors almost certainly plays a role in generating and shaping motor outputs during crawling as it does for locomotor behaviors in other animals (reviewed: Stein et al., 1999; Pearson, 2000; Büschges, 2005). I have gathered preliminary data that suggest spiking in individual pressure-sensitive and nociceptive mechanosensory neurons (the P and N cells, respectively) is sufficient to reset the DA-induced fictive crawling rhythm in a single isolated ganglion. Spiking in the touch-sensitive neurons (T cells) had no noticeable impact on DA-induced fictive crawling. If I were to continue with this line of research, I would attempt to stimulate P and N cells in isolated but intact CNS preparations during coordinated DA-induced fictive crawling to determine if interrupting the activity of a single CPG has any effects on their intersegmental coordination. My prediction is that it would because: 1) stimulation of the body in nearly-intact leeches perturbs the membrane potential of R3b-1 (Esch et al., 2002); 2) My preliminary data indicate that intracellular stimulation of a P cell perturbs R3b-1. These findings suggest that a strong sensory input to the CNS affects both the local crawl CPG and a part of the command-like system that governs crawling globally (Chapter 4). By understanding better how sensory information interacts with local and higher-order systems, insights could be gained as to how sensory systems interact with central pattern-generating networks in general.

Another important area for further study of crawling would be to identify neurons that comprise the CPG for crawling. Doing so would open up myriad future investigations into the specific cellular mechanisms of this flexible CPG and the higher-

order and/or sensory systems that interact with the crawl CPG. I briefly attempted to identify members of the CPG for crawling, but was unsuccessful. I did, however, begin developing methods for locating candidate neurons more easily based upon already-established VSD imaging techniques (Cacciatore et al., 1999). The VSDs allow the membrane potentials of many neurons to be monitored simultaneously during fictive crawling. Once fictive crawling is activated (electrically or chemically), cells are imaged and phase relationships are calculated for each visible neuron (recorded optically). The reference signal for calculating phase of the optical data is the crawl-monitor motoneuron (DE-3) in the dorsal posterior nerve. Cells that are phase-locked and significantly coherent with crawl motor outputs are candidate members of the CPG. To determine whether or not a candidate cell is a rhythm-generating element for crawling, traditional intracellular electrophysiological recording techniques are used while optical recordings are made. If perturbing the rhythmic activity of a given candidate neuron, *via* injecting depolarizing or hyperpolarizing current, resets the rhythmicity of fictive crawling, that cell is likely to be part of the CPG.

As stated above, my thesis research has elucidated that DA is a potent modulator involved in promoting crawling behavior at levels of the cephalic (Chapter 4) and segmental ganglion (Chapter 2). How the DA neurons become activated to release DA has yet to be determined. Briefly, I began such studies by intracellularly recording from individual DA-synthesizing neurons in the cephalic ganglion (Crisp et al., 2002) to determine their synaptic relationships. During my recordings, I depolarized (i.e., activated) individual DA neurons and monitored fictive motor outputs, however, I did not observe fictive crawling induced by stimulation of the dorsal-lateral DA neurons of the SEG. One interpretation of this result is that a different sub-population of DA neurons releases DA in a way that promotes crawling or that multiple DA neurons need to be

activated simultaneously to promote crawling. Another worthy experiment would be to simultaneously stimulate individual DA neurons while stimulating R3b-1 and monitoring fictive motor outputs. If R3b-1 stimulation were found to induce fictive crawling exclusively, this result would support that neuronal release of DA has the ability to bias the CNS to crawl. Although I was unable to show the direct link between identified DA cells and the initiation of crawling, I was able to make some of the first electrophysiological recordings from the DA cells in the cephalic ganglion. I elucidated their individual morphology (example, Fig. 5-2) by ionophoretically injecting them with Neurobiotin using the methods of Gilchrist et al. (1995) and demonstrated that although they are relatively small cells, compared to other leech neurons (~10 μ m), they have the ability to generate spiking activity. My hope was to identify cephalic DA neurons that project to the segmental ganglion. None of the dorsal lateral DA neurons projected beyond the cephalic ganglion, however, they did establish rich arbors within the SEG, spanning the length of the SEG neuromeres (Fig. 5-2).

I was also involved in a host of pharmacological studies to alter the release, uptake or synthesis of DA in an effort to study how DA perturbation could be linked to locomotor alterations or deficits. One type of experiment I conducted involved treating individual segmental ganglia with Bupropion, which blocks DA reuptake and essentially increases endogenously released DA levels. I treated several isolated ganglion preparations with Bupropion and observed fictive rhythmic activity that resembled crawling, as recorded in the dorsal posterior nerve. I did not, however, determine if the circular and longitudinal motoneurons were bursting anti-phasically or if ventral and dorsal longitudinal motoneurons were bursting concurrently. If endogenously released DA can, indeed, be demonstrated to promote crawling behavior, such results would provide some of the first data to date, in any animal, that directly links a particular

behavior with a neuronal release of DA. Another parallel line of pursuit I took was to interrupt DA signaling by treating intact leeches with the D2-like DA receptor antagonist haloperidol and then observing their overt behaviors (Puhl et al., 2006). Briefly, I learned that applying haloperidol to the pond water in concentrations of ca. 40 μM for 1-2 weeks interrupted overt swimming. Curiously, my analyses did not show any notable interruptions to crawling, perhaps because I was not targeting the appropriate type of DA receptor. Furthermore, these treatments appeared to reduce the amounts of DA within the finer processes of the cephalic and segmental DA-containing neurons (visible *via* immunocytochemistry and glyoxylic acid fluorescence), suggesting some sort of disruption to auto-regulatory feedback systems regulating DA synthesis. As a follow-up to my imaging studies, the DA levels of control and haloperidol leeches should be quantified using HPLC or some other reliable quantification method.

Functional recovery of crawling after major CNS injury: An exciting new horizon

As part of verifying that descending signals from the cephalic ganglion are necessary for overt crawling, I conducted a pilot study where I transected the connectives between the SEG and M1 in otherwise intact leeches and observed their behaviors. The transections had no gross effects on the expression of the local-bend and shortening reflexes or swimming. As my data from Chapter 3 would predict, however, the SEG/M1 transections completely interrupted crawling behavior (schematic; Fig. 5-3A). Much to my surprise, however, 4-10 days after such transections 90%+ of leeches recovered their ability to crawl (Fig. 5-3B,C). The recovered crawling was nearly indistinguishable from crawling exhibited by leeches that received a sham surgery (i.e., received the surgery with no transection) or no surgery at all. The results described

below were presented at the 2010 Society for Neuroscience meeting (Mesce et al., 2010).

How this recovery might be achieved and some preliminary data to address a potential mechanism are briefly outlined here. The first hypothesis that was tested was that M1 possesses some sort of special property that takes over for crawling when R3b-1 signals are absent. To test this hypothesis, the connectives between M1/M2 were transected in leeches that had recovered their ability to crawl after an SEG/M1 transection (schematic; Fig. 5-3D). Upon this 'secondary' transection, crawling behaviors were immediately interrupted again. Swimming, local bending and reflexive shortening behaviors were unaffected. Amazingly, within 6-14 days, most of the leeches again regained their ability to crawl (Fig 5-3, E,F). This suggested that M1 did take over the function of driving crawling behavior (i.e., acted as the "lead ganglion"), however, upon its removal another ganglion, presumable M2, became the new lead ganglion. These results also established that M1 likely does not contain some special property that the other ganglia do not. Future studies will verify the lead ganglion hypothesis by performing a tertiary transection between M2/M3 on animals that have recovered from previous SEG/M1 and M1/M2 transections. I predict animals receiving a tertiary M2/M3 transection will experience an interruption in crawling behavior with eventual recovery.

Another hypothesis that was tested was whether the terminal compound ganglion (TG) plays any role in the recovery of crawling behavior after transection of the CNS. In Chapter 4, I established that the TG is not necessary for crawling or the intersegmental coordination of the crawl CPGs. To test this hypothesis further, the M21/TG connectives were transected several weeks after leeches had received their initial SEG/M1 transection and were crawling again. In recovered leeches, transection of the TG resulted in no loss of crawling. In these TG-transected leeches, if the M1/M2

connectives were transected, they recovered their ability to crawl and the time-course for recovery was similar to that of leeches with connections to the TG intact. Thus, the TG does not appear to play a role in the recovery of crawling.

The ability of a leech to recover its crawling behavior after removal of descending cephalic information (i.e., R3b-1 and perhaps other descending units) likely arises by one of 3 mechanisms, although many other possibilities may exist. Our lab is actively investigating these possibilities. The first is that the neuronal fibers simply regenerate. Our observations have determined that this almost certainly is not the case. The second possibility is that perhaps the projecting portion of R3b-1 does not degenerate after being separated from its soma. If a spike initiating zone for R3b-1 is present below the transected area or if one or more new spike initiating zones form in response to the transection, R3b-1 signaling may be restored to the oscillators posterior to the transection. Last, upon cessation of R3b-1, the processing of sensory information by the crawl CPGs may become altered so that they become more sensitive to stretch receptor and/or other mechanosensory neuronal inputs.

Summary and significance to work in vertebrates

My thesis research has elucidated some of the underlying organization of the distributed neural networks that control crawling in the medicinal leech. I have provided evidence of a specific behavioral role that DA plays in controlling locomotion. I established a clear relationship between higher-order CNS regions and distributed pattern-generating neural networks and proposed a novel mechanism for how they interact to generate coordinated and productive locomotor movements for a behavior that possesses a high degree of flexibility. Last, I characterized further an identified higher-order neuron that makes multi-dimensional contributions to controlling locomotor

behaviors. A final thought that I will explore in my thesis is how do my contributions potentially benefit our understanding of motor control in more evolutionarily advanced animals such as walking vertebrates?

My work establishing that DA acts to promote crawling behavior creates an interesting parallel with data reported for walking animals. A consequence of the death of DA-releasing neurons in the basal ganglia, human motor diseases like Parkinson's clearly establish the integral roles of DA in the generation of movements (Davie, 2008). Studies of the rat have implicated DA in the generation of slow walking-like neural activity as well (Kiehn and Kjaerulff, 1996; Barriere et al., 2004). Thus, my research that elucidates DA's role in the activation of locomotor CPGs may help to provide a better understanding of the interactions between dopaminergic and locomotor control systems. My results showing that DA specifically acts at both the segmental CPG and the cephalic ganglion may shed new light on how DA regulates motor behaviors in higher animals. For example, if DA is released systemically into the blood stream, it can act as a neurohormone to organize the cephalic ganglion and all of the segmental ganglia so that they all enter into a crawl mode. By understanding more global strategies for how DA networks instruct and organize specific motor activities, perhaps new treatments for diseases such as Parkinson's can be developed.

My work in Chapters 3 and 4 propose a novel mechanism for coordinating CPGs that generate a flexible locomotor behavior that incorporates higher-order command-like neurons. Such information may lead to a general schema of how movements are coupled or can become uncoupled. For example, when we walk, our arms swing naturally in opposition to our legs; these movements occur with little or no conscious thought. While we are walking, however, we can easily uncouple the movements of our arms from our legs without detriment to walking. Similar results have been shown in the

decerebrate cat, where the activities of front and hind limbs can be uncoupled using a split treadmill apparatus (Akay et al., 2006). Thus it is clear that descending commands are vital for such coordination.

Final thoughts on the command neuron concept

The original view of a command neuron (or command system) was that there was 'dedication' to a single aspect of a single behavior (Wiersma and Ikeda, 1964; Bowerman and Larimer, 1974). However, such a one-cell, one-function, paradigm is one aspect of the original concept unlikely to hold up, whether it be in insects (Ritzmann et al., 1980, Nolen and Hoy, 1984, Böhm and Schildberger, 1992), leeches (Brodfuehrer et al., 1995; Brodfuehrer and Thorogood, 2001; Kristan et al., 2005; Mesce et al., 2008; Chapter 4) or vertebrates (Deliagina et al., 2002; Dubuc et al., 2008; Jordan et al., 2008; Zelenin, 2011). Instead, animals appear to employ a mixture of cells that are arranged across a spectrum of multi-functionality and respond differently in altered contexts. My work has extended this idea by showing that a single neuron can affect multiple behaviors in multiple ways, including the novel dimension of being necessary for intersegmental coordination. By having a wide array of control elements with command-like features that are coupled to CPGs and are sensitive to modulatory and sensory inputs, animals are able to tap into a rich tool-box from which dynamic and adaptable behaviors can be generated.

Concluding remarks

During my dissertation research I made a number of discoveries that have advanced our knowledge of the underlying organization and cellular mechanisms that generate crawling in the medicinal leech. My discoveries add to an already vast corpus of information about the neural underpinnings of motor behaviors in the leech (reviewed:

Kristan et al., 2005) and also shed light on potential mechanisms that may generate flexible locomotion in other animals such as walking vertebrates. Furthermore, my work may help to develop treatments for Parkinson's disease as well as debilitating spinal cord injuries. It may also aid in the development of more advanced prosthetic or robotic devices to assist or enhance a person's ability to generate movements. Last, my research may advance our overall understanding of complex control schemes that govern rhythmic behaviors in general. To gain this level of insight, my studies explored the behavior of an intact animal down to the level of an individual neuron, a journey made possible because of the extreme experimental accessibility of the relatively simple medicinal leech.

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Figures

Figure 5-1: Preliminary data (N=1) showing potential synaptic target neurons of R3b-1 in ganglion M10 of an intact CNS preparation. **A**, Image of voltage sensitive dye (VSD) fluorescence of the ventral aspect of M10 (see schematic below for orientation of the ganglion). The ellipses (labeled a-o) identify individual neuronal somata of cells that were in the plane of focus. Colored somata had optical activity that was significantly coherent (i.e., phase locked) with 1 Hz, 3 nA current pulses injected into R3b-1 [bottom trace in **B**]. The colormap (right) denotes the phase of the optical activity relative to the membrane potential of R3b-1. The CV motoneuron somata are denoted in the VSD image as well as in the schematic (filled somata). **B**, $\Delta F/F$ traces of the somata denoted in **A**. An asterisk next to the trace denotes optical activity that was significantly coherent with the membrane potential of R3b-1 (bottom trace). Vertical scalebars denote 1% $\Delta F/F$.

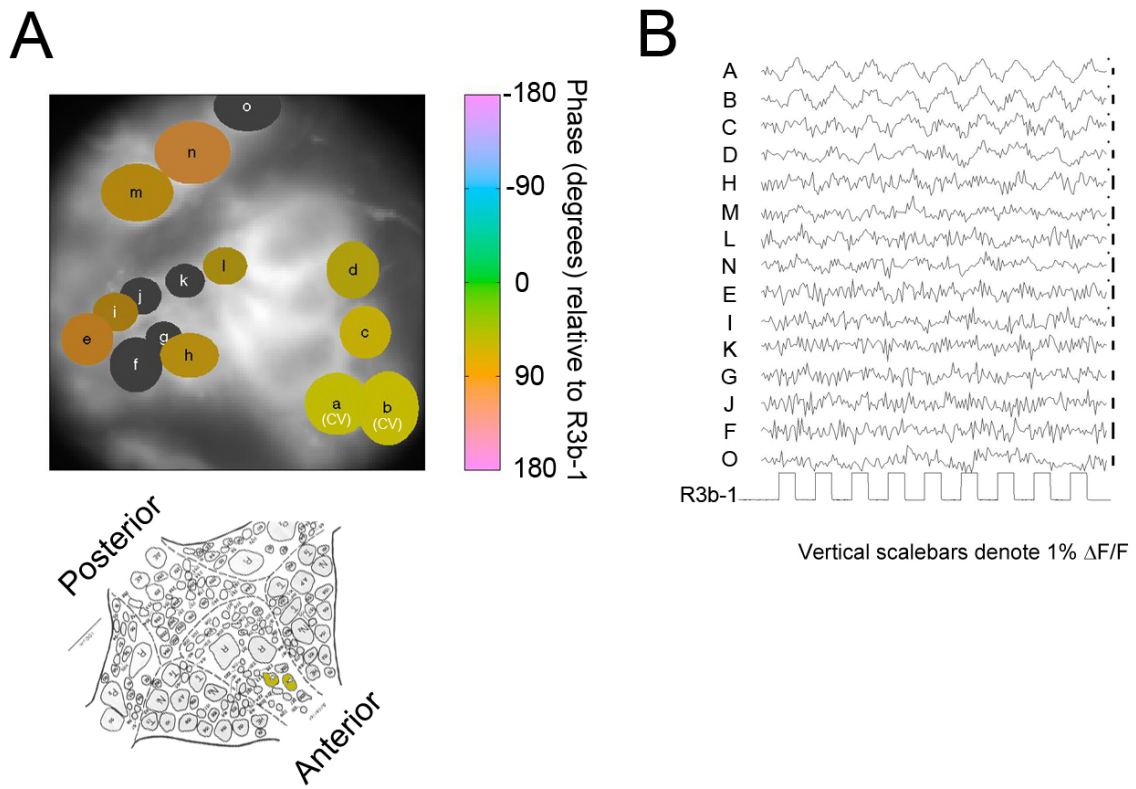


Figure 5-1: Voltage-sensitive dye imaging of potential synaptic targets of R3b-1.

Figure 5-2: Intracellular Neurobiotin fill (NB) and tyrosine hydroxylase immunocytochemical labeling (TH; inset) of a dorsal-lateral DA-containing neuron within the SEG of an adult medicinal leech. The Neurobiotin was visualized according to the methods described in Chapter 4. Scalebar is 100 μ m.

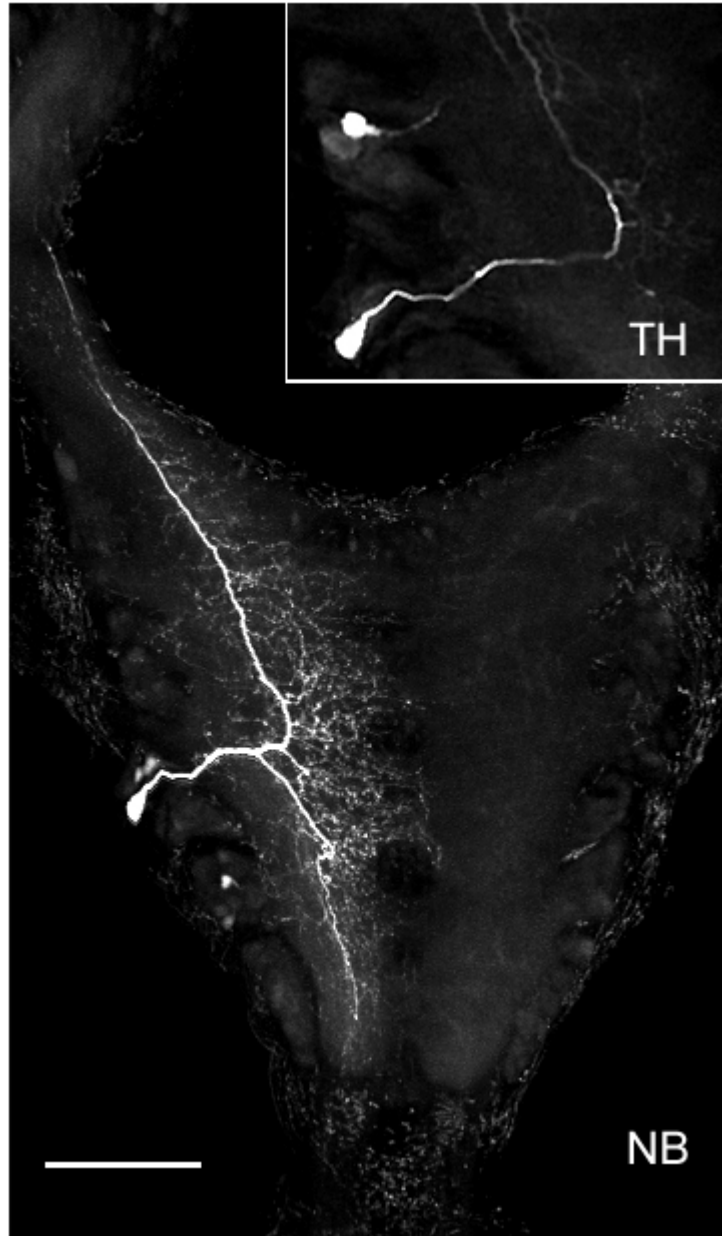


Figure 5-2: Morphology of a dorsal-lateral DA-containing neuron within the SEG.

Figure 5-3: Transection of the interganglionic connectives causes interruptions in overt crawling behaviors of intact leeches, however, these animals recover the ability to crawl over time. **A-C**, Transection of the SEG/M1 connectives leads to an immediate interruption in crawling behavior. After 3-10 days post transection, spontaneous crawling returns. **A**, Schematic showing location of the SEG/M1 transection. **B**, Bar graph depicting the percentage of transected animals that showed spontaneous crawling behavior during an 8-minute observation session. Day -1 was prior to the transection surgery. Numbers inside the bars denote sample sizes. **C**, Graph showing the average number of crawl cycles observed before (day -1) and after SEG/M1 transection. **D-F**, Transection of either the M1/M2 or M2/M3 connectives in animals that have recovered from a previous SEG/M1 transection reintroduces interruptions to crawling. **D**, Schematic showing the location of the initial SEG/M1 transection (1) and the secondary M1/M2 or M2/M3 transection 38 days after the initial transection (2). **E**, Bar graph depicting the percentage of transected animals that showed spontaneous crawling behavior during an 8-minute observation session. Day -1 was prior to the secondary transection surgery. Numbers inside the bars denote sample sizes. **F**, Graph showing the average number of crawl cycles observed before (day -1) and after the secondary M1/M2 or M2/M3 transection.

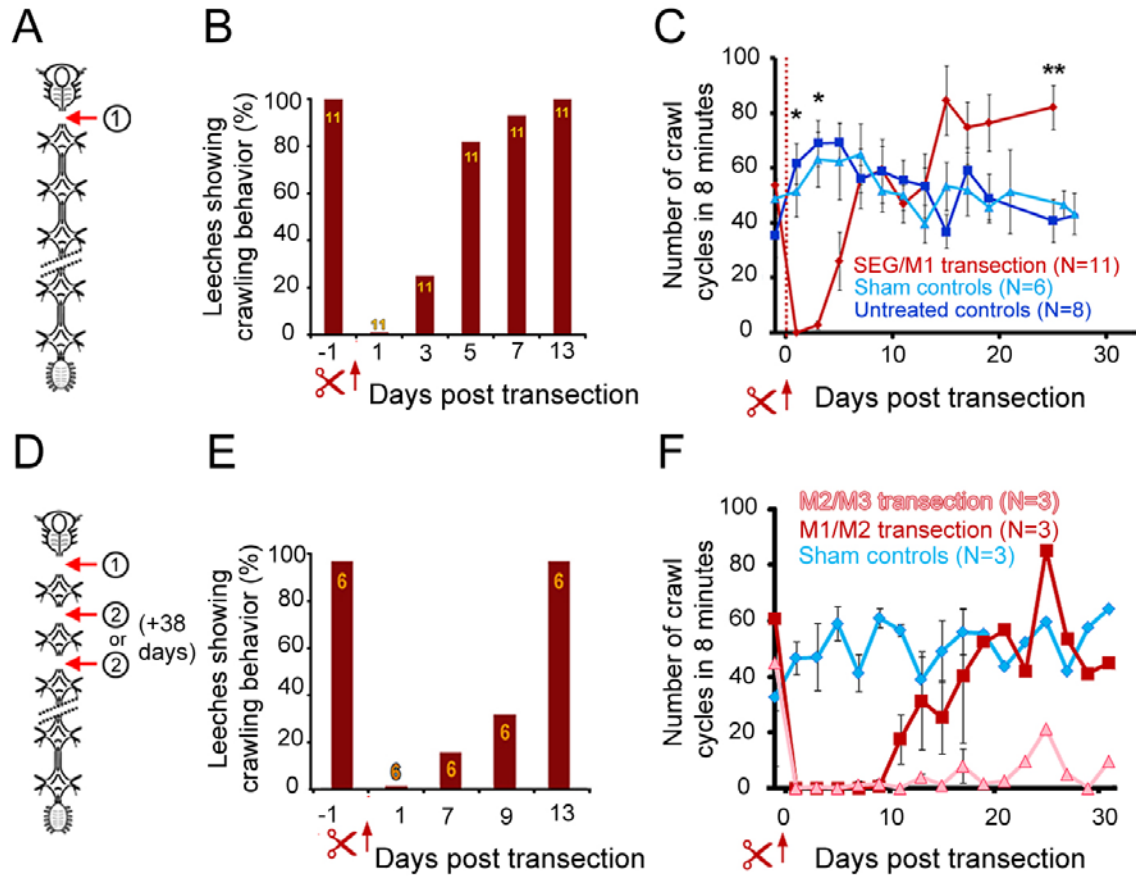


Figure 5-3: Recovery of overt crawling after transection of the interganglionic connectives.

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