The Anatomic Connectome and Functional Dissection of the *Caenorhabditis elegans* First-Stage Larvae (L1) Motor Circuit

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

During development, neural networks exhibits anatomic and functional modifications. Yet their activity patterns change remains poorly understood. The neural circuit controlling locomotion of *C. elegans* undergoes major reconstructions at the end of first-larval (L1) stage. In adults, the ventral/dorsal body wall muscles are symmetrically innervated by cholinergic and GABAergic motor neurons (MNs). Cholinergic MNs innervating ventral muscles are not derived during early L1 stage, yet the animals can still generate undulation. This study addressed how L1 *C. elegans* produces undulation and the mechanism underlying muscle innervations. We demonstrated that the activities of dorsal cholinergic MNs are required for both dorsal and ventral muscle contraction, and the GABAergic MNs mediate only ventral muscle relaxation. This indicated a differential synaptic wiring between MNs and muscles in L1 to permit undulation. Through this comparative analysis between juvenile and adult locomotion, we hope to provide insights on principles of behavioral adaptation during sensorimotor development.
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Specific Contribution

Fig 1-1, 1-2, 1-3, 2-1 was prepared by Sihui Guan. Some diagrams were adapted from published studies, reference and citation were included in the figure legends.

Fig 2-2, 2-3, 2-4, 2-5, 2-6, 2-10, 2-11: All experiments and figures were prepared by Sihui Guan

Fig 2-7: Panel A was prepared by Sihui Guan. Panel B and C were prepared by Yangning Lu.

Fig 2-8: Panel A and B were prepared by Sihui Guan. Panel C was prepared by Yangning Lu.
1. Introduction

1.1. Animal behaviors represent an integrated and coherent output upon the interaction between neural circuits and the environment

1.1.1. Sensorimotor neural networks govern behavioral output

Both vertebrates and invertebrates generate a rich repertoire of motor actions in response to their environment. Sensing specific environmental stimuli, timely decision-making, executing and switching between appropriate motor behavioral responses allow animals to achieve the ultimate goal in evolution, survival and reproduction.

Locomotion is generated by networks of neurons that initiate and/or maintain rhythmic activity patterns entrained by sensory feedbacks. It is a coordinated motor output resulting from intricate interactions between the pattern generating circuit and different feedback mechanisms that fine-tune the rhythm and pattern (Briggman and Kristan, 2008; Goulding, 2009; Mu and Ritzmann, 2008; Rossignol et al., 2006; Zhen and Samuel, 2015). Despite the morphological and numerical differences in their anatomy, the functional architecture of all examined sensorimotor networks contains three major components: stimulus sensor, neuronal pattern generator and motor output executor.

In a simplified schematic (Fig 1-1), a group of neurons, which has the ability to generate and maintain rhythmic activity patterns, activate motor neurons in an appropriate sequence that give rise to a seamless and dynamic pattern of coordinated muscle activity. Information from the environment gathered by sensory neurons, is projected to the rhythmic generating neurons to influence the choice and characteristics of the motor sequences (Rossignol et al., 2006).
Generating cohesive movements involve multiple rhythm generating circuits and hence their coordination. For example, in rodents and cats, separate motor units in the spinal cord give rise to rhythmic and patterned muscle contraction to drive the left and right limbs, respectively. During walk, contralateral inhibitory commissural interneurons interact with the left- and right-limb motor modules to secure a left-right alternating activation pattern. Within the same limb, motor neurons that govern the activity pattern of the flexor and extensor muscles must activate alternatively, which involves several groups of inhibitory interneurons (Goulding, 2009; Grillner, 2006; Kiehn, 2011; Kiehn et al., 2010; Rossignol et al., 2006).

Multiple feedback mechanisms shape the rhythm and pattern of motor CPGs. In both vertebrates and invertebrates, descending and ascending pathways provide these feedbacks. Descending signals from the supraspinal brain region integrate multiple sensory signals (visual, auditory, etc.) to modulate gait duration and pattern (Grillner, 2006). Ascending signals, including local signals from the cutaneous and proprioceptive afferents, allow the adjustment for proper limb placement during each movement cycle (Rossignol et al., 2006).

Hence, even the most simplified motor circuit utilizes various layers of dynamic sensorimotor interactions to generate motor behaviors. Recent advances have begun to uncover the molecular, cellular and circuit mechanisms for each of these pathways in various animal models (Grillner, 2006; Kiehn, 2006; Kiehn et al., 2010; Rosenbaum et al., 2010; Zhen and Samuel, 2015). Yet it remains an intriguing challenge to integrate our current knowledge into a realistic model of locomotion generation.
Figure 1-1. A diagram of the sensorimotor system in animals.
1.1.2. Rhythmic locomotion is generated by dedicated motor circuits

1.1.2.1. Central Pattern Generators (CPGs)

Neural networks that give rise to coordinated and rhythmic neuronal activities define various motor behaviors (Dickinson, 2006; Marder and Bucher, 2001). Core components of these neural networks are often referred to as the Central Pattern Generators (CPGs). Like the name suggested, CPGs can spontaneously generate and sustain the rhythmic membrane potential oscillation in the absence of sensory inputs (Calabrese, 1995; Grillner, 2006; Marder and Bucher, 2001).

In the mid 1900s, an emerging notion that the generation of motor outputs is governed by pattern-generating neural networks began to challenge the long-standing doctrine that motor activities arise passively as a complex chain of reflexes (Brown, 1911; Wilson, 1961; Wilson, 1966). The earliest evidence for the CPG property of motor circuits came from studies on the locust (Wilson, 1961): when both the ascending and descending sensory afferents were severed surgically, the body could still produce a similar muscle contraction pattern to those in flight. A flight-inducing cue (such as a continuous stimulation of the wind-sensitive head hairs) or an direct electrical stimulation of the thoracic ganglia was still able to trigger the oscillating motor activity in the deafferented locust, albeit the oscillation frequency was reduced and the cycle time was less accurate (Wilson, 1966). These observations strongly suggest that the motor ganglia in the locust are capable of producing fictive motor output in the absence of sensory inputs, whereas the sensory feedback refines the maneuver control by influencing the frequency and amplitude of the CPG pattern (Wilson, 1961).
These findings inspired studies in other animal models, which cumulatively began to establish the founding principle and the mechanism for motor pattern generation across species. For example, in the lamprey, the activity of an isolated spinal cord without sensory or descending inputs had been examined. The deafferented preparation exhibited a fictive motor pattern (Cohen and Wallen, 1980), where the sequential activation and the left-right alteration of the series of CPGs residing along the spinal cord was maintained like in the intact animals (Cohen and Wallen, 1980; Sigvardt and Miller, 1998).

To date, neural networks with CPG properties have been documented in many nervous systems, and animals are found to be endowed with multiple CPGs for dedicated motor functions (Calabrese, 1995; Grillner, 2006; Kiehn, 2006; Kupfermann and Weiss, 2001; Marder and Bucher, 2001). In the following sections, I will describe a few studies that addressed the underlying mechanisms for rhythm and pattern generation.

1.1.2.2. Basic mechanisms for pattern generation by CPGs

A CPG may consist of single or multiple pacemaker neurons, or of a group of interconnected neurons that each on their own does not have intrinsic pacemaker properties (Calabrese, 1995; Grillner, 2006; Marder and Bucher, 2001). In a pacemaker neuron driven CPG network, a single, or several electrically coupled neurons could act as the core oscillator, providing rhythmic inputs to other neurons in the network (Canavier et al., 1991; Kiehn et al., 2000). Most CPGs, however, are driven by a network of neurons that generate oscillating activity through a combination of synaptic connectivity and endogenous membrane properties (Friesen, 1994).

The pacemaker CPG system
Pacemaker neurons exhibit intrinsic oscillating electric activities. A combination of ion channels expressed by these neurons endow such an intrinsic property, and determines the relative duration of each phase of the oscillation cycle (Calabrese, 1995; Grillner, 2006). There are three general key features to their rhythm-generating property.

Firstly, voltage-dependent ion channels are essential for the depolarization phase. Voltage-gated Na\(^+\) channels, the NMDA channels, and the L-type Ca\(^{2+}\) channels are sequentially engaged to establish the rising and maintenance phase of a plateau potential (Harris-Warrick, 2002; Wallen and Grillner, 1987), which allows for action potential bursting (Harris-Warrick, 2002). Secondly, membrane-hyperpolarizing channels terminate the depolarization and action potential bursting. Calcium-activated potassium channels typically function to facilitate membrane hyperpolarization (Pape, 1996).

Thirdly, a slow and autonomous depolarization current terminates of the hyperpolarization phase and initiates another round of depolarization. This so-called hyperpolarization-induced depolarizing current is carried out by low-voltage gated calcium channels in many cases, which is a crucial property of many pacemaker neurons (Angstadt et al., 2005; Bertrand and Cazalets, 1998; Harris-Warrick et al., 1995; Matsushima et al., 1993; Satterlie, 1985).

An extensively studied example of pacemaker CPG is the pyloric rhythm generating circuit of the crustacean stomatogastric system. A group of electrically coupled neurons, the AB and PD cells, constitute the main intrinsically bursting neurons governing the pyloric rhythm, while additional neurons are conditionally recruited in accordance to modulatory signals (Eisen and Marder, 1982; Marder and Bucher, 2001; Nusbaum and Beenakker, 2002). Bursting generated by the AB and PD neurons has a
given frequency (similar to a fictive locomotion pattern), while synaptic inputs from sensory and interneurons alter the bursting frequency and phase duration (Combes et al., 1999; Nusbaum and Beenhakker, 2002). The interaction between the pacemaker AB/PD neurons and the modulatory neurons gives rise to an optimized pyloric pattern for the environment.

**The coupled oscillator CPG system**

In many systems, rhythmic oscillations originate from a network of neurons that do not burst on their own. In many cases, rhythmic bursting of the network results from reciprocal inhibition between neurons of the network with certain intrinsic membrane properties.

First suggested by Brown as a model (1911), reciprocal inhibition has become a major mechanism for rhythm generation (Satterlie, 1985). Brown’s model, called the “half-center oscillator” (Fig 1-2), consists of as simple as two neurons that impose inhibition onto each other; one or both may receive excitatory inputs to initiate the oscillator (Brown, 1911). Brown also recognized that to prevent two mutually inhibitory neurons to act as simple switches that synchronously turn each other off, these neurons should have specific membrane and synaptic properties (Brown, 1911; Friesen, 1994). There have since been numerous experimental supports for such a model (Grillner, 2006; Marder and Bucher, 2001; Wang and Rinzel, 1992).

Synaptic properties between coupled neurons play a critical role in underlying the reciprocal inhibition-dependent membrane oscillation. Their synaptic connections may exhibit adaptive properties: neuron A, which inhibits neuron B, gradually reduces the spike frequency even when receiving constant excitatory input so that the inhibitory
neurotransmitter release decays over time. This results in an elevation of neuron B’s membrane potential, releasing it from inhibition, and activated neuron B will in turn inhibit neuron A (Marder and Bucher, 2001; Skinner et al., 1994). The activation of neuron B follows a similar adaptive mechanism, resulting in a cyclic anti-phasic oscillation between neuron A and neuron B (Calabrese, 1995; Friesen, 1994; Grillner, 2006; Skinner et al., 1994).

Membrane properties of these synaptic coupled neurons, such as the post-inhibitory rebound, ensure rhythm generation and maintenance (Calabrese, 1995; Friesen, 1994). Two mechanisms account for the activation of these neurons: a hyperpolarization-activated inward current that slowly builds up during the inhibition phase, and a low voltage-activated calcium current that is activated at the termination phase of the synaptic inhibition (Calabrese, 1995; Friesen, 1994; Grillner, 2006). Both currents may contribute to the initiation of the depolarization phase of the neuron under inhibition. Upon its activation, it begins to inactivate the other neuron (el Manira et al., 1994; Grillner, 2006).

In summary, a dynamic control of membrane potential, regulated by synaptic adaptation and membrane conductance, is critical for pattern generation ability of non-pacemaker CPG networks to drive rhythmic motor behaviors.
Figure 1-2. A basic model of rhythmic oscillation is generated by reciprocal inhibition between at least two non-bursting neurons.

Top panel, when uncoupled, two neurons that are not pacemakers fire separately with no correlation. Bottom panel, when two neurons exert reciprocal and mutual inhibition, they can fire in alternating bursts. Adapted from Marder and Bucher, 2001.
1.1.3. Activation, inhibition, and modulation of motor circuit CPGs

How do the motor circuit network modulation and coordination take place? Specifically, what determines the selection, recruitment, activation, termination and modulation of the appropriate CPGs to generate desired behaviors in a given environment? Although CPGs intrinsically generate rhythmic activity patterns in the absence of sensory inputs, these inputs from descending pathway or local afferent feedbacks are critical for modifying and optimizing the pattern. Two main mechanisms, the recruitment of specific CPGs and the modulation of the operating CPGs, ensure the generation of appropriate and adaptive motor responses to the environment.

1.1.3.1. Activation and termination of CPGs by the descending control

In vertebrates, multiple motor CPG units that control different limb muscles reside along the spinal cord, whereas the basal ganglia command the spinal CPGs (Grillner, 2003; Grillner et al., 2005). In particular, the recruitment and activation of specific spinal cord CPG modules rely on the descending control from the basal ganglia. The descending projections from the palladium exert tonic inhibition of the CPG activity. Should a motor behavior be activated, the striatal neurons that project onto the basal ganglia selectively inhibit certain palladium neurons, disinhibiting the spinal cord CPGs to elicit a rhythmic motor pattern. The striatal neurons also receive signals from cortical regions such as the neocortex or thalamus, which allow them to integrate higher order sensory information to optimize the motor response (Grillner et al., 2005).
In invertebrates, projection neurons from the central ganglia also act as switches to turn on and off CPG motor modules for specific body segments (Kupfermann and Weiss, 2001). These neurons, serving command-like functions, typically belong to structures functionally analogous to that of the vertebrate brainstem. In the leech, two types of cephalic projection neurons (Tr1 and Tr2) have been shown to influence the descending decision-making pathway to initiate swimming. Their somata located in the subsophageal ganglion, and their descending axons innervate the swim CPGs that reside in the ventral nerve cord. The activation of Tr1 provided excitatory drive to segmental swimming gating-neurons, which, via direct synaptic connections to swimming CPGs, triggered swimming episodes (Brodfuehrer and Friesen, 1986). In contrast, the stimulation of Tr2 caused the termination of swimming without direct synaptic connections to the swimming gating-neurons or swimming CPGs, but rather, to another set of intersegmental neurons (Cell 256), whose activation may stop the swimming CPG (Brodfuehrer and Friesen, 1986). Several direct synaptic targets of Tr2 on segmental nerve cord have been identified; upon stimulation by Tr2, they induced the termination of swimming (Taylor et al., 2003). It was proposed that these target neurons are engaged in the inhibition of the swim CPG or swimming gating-neurons, but more experimental evidence is required to confirm this hypothesis.

1.1.3.2. Modulation of the CPG activity patterns

CPGs establish rhythmicity of motor behaviors, but the length and phase of the motor behaviors are under constant modification (Dickinson, 2006). This is achieved through
regulation of CPG activities, via neuromodulators or direct synaptic inputs to CPG components (Briggman and Kristan, 2008; Dickinson, 2006; Faumont et al., 2005).

1.1.3.2.1. Neuromodulator

Neuromodulators, received either from a local source (secreted from neurons nearby) or hormonally (via circulation), may regulate the CPG network dynamics through modifying a CPG neuron’s intrinsic membrane property or synaptic properties between neurons within CPG network (Dickinson, 2006; Faumont et al., 2005). From monoamines to neuropeptides, a variety of substances have been shown to change the rhythm and strength of CPG networks. Monoamines such as dopamine and serotonin have been shown to modulate network properties in several organisms, often in a conserved fashion. In the lamprey, dopamine and serotonin exert significant influence on the length of the fictive swimming CPG cycle. 5-HT modulates the gating property of calcium-dependent potassium channels that hyperpolarize the neuron after the action potential train. The modulation leads to an attenuation of outward potassium current during the hyperpolarization phase, resulting in a prolonged hyperpolarization period and CPG cycle. In contrast, dopamine acts to reduce the calcium entry during the depolarization and bursting periods, which subsequently reduces the calcium dependent outward current of potassium. Therefore, these two mechanisms exert complementary actions on the afterhyperpolarization phase of motor neurons and some CPG neurons, leading to changes in the cycle period (Kemnitz, 1997; Schotland et al., 1995; Van Dongen et al., 1986; Wallen et al., 1989). Serotonergic modulation of motor behaviors is also present in other vertebrates. For example, in an adult Zebrafish spinal cord
preparation, bath application of NMDA stimulates rhythmic and fictive swimming patterns. Stimulation of endogenous serotonin release leads to a decreased burst frequency of motor neurons, and a decrease of the fictive swimming. Serotonin exerts such an effect by potentiating the activity of inhibitory neurons of the swimming CPG (Gabriel et al., 2009).

1.1.3.2.2. Synaptic modulation

Sensory feedbacks exert numerous effects on CPG activities, such as altering the frequency, rhythm of CPG neurons and the amplitude of muscle discharges. Direct synaptic inputs from interneurons and sensory neurons to the CPG motor unites can mediate many of these effects. In an extensive review (Rossignol et al., 2006), Rossignol and colleagues discussed how sensory inputs shape the property of motor CPGs in vertebrates through dynamic synaptic interactions between CPGs and sensory neurons from both ascending afferents and descending projections.

Key sensory feedbacks on vertebrate motor CPGs include the cutaneous feedback, proprioceptive input from muscles or tendons, and the descending interneuron input from the supraspinal region. In cats and rodents, tonic cutaneous stimulations either enhance (Pearson and Rossignol, 1991) or inhibit (Viala et al., 1978) the CPG activity and the fictive locomotion rhythm. On the other hand, proprioceptive feedbacks from muscle afferents and tendons were found to entrain the rhythm of locomotion through mechanisms that have not been fully dissected. In cats, during stance, the proprioceptive afferents from the limb extensor can adjust the amplitude of limb muscle activities to
alter the speed of locomotion. Stimulation of specific muscle afferents can reset the cycle through multiple circuit activities (Reviewed in (Rossignol et al., 2006).

The sensory pathway includes the primary neurons, descending command neurons and segmental spinal cord interneurons. Presynaptic potentiation and inhibition between components of the sensory pathway modify the sensory inputs to the motor CPGs. Components of the sensory pathways can also directly affect the excitability of the CPG neurons, as well as of motor neurons, to alter the properties of the phase and pattern of the motor outputs. These multi-layer modulations are proposed to permit the versatility of behavioral patterns in animals (Rossignol et al., 2006). In summary, a CPG unit is subjected to multiple functional modulations and a combinatorial effect ultimately gives rise to different behavioral outcomes.

1.1.4. Cohesive movement relies on coordinated activities of multiple CPGs

Multiple spinal cord motor CPGs enable vertebrate locomotion and specific CPG modules are dedicated for specific muscle groups (Kiehn et al., 2010; Marder et al., 2005). In undulators, animals that swim or crawl, dedicated CPG modules are responsible for bending of each body segment (Grillner, 2006). In limbed animals, dedicated CPGs are responsible for contraction and relaxation of the flexor and extensor muscles in each limb (Kiehn et al., 2010; Tresch et al., 2002). Rhythmic activation of different CPG units with proper temporal coordination is necessary to generate the muscle synergy that enables coordinated limb movement, propulsion of body mass, and directional locomotion (Briggman and Kristan, 2008).
1.1.4.1. Undulation

Undulatory motor behaviors involve a chain of body segment contraction and relaxation that forms a propagating bending waveform (Grillner, 1985, 2003) (Grillner, 1985, 2003; Bem et al, 2003). Animals such as the leech, *Xenopus* tadpole and lamprey produce such propagating waveforms, accompanied by a left-right bending alternation, to generate the propelling force for forward and backward movement (Marder and Rehm, 2005).

Forward and backward movement in undulators can be regarded as a series of segmental CPG oscillation, coupled by phase offsets in either an anterior to posterior temporal direction, or vise versa. In many undulators, such as the lamprey, each body segment contains a CPG that is capable of generating rhythmic motor neural activity pattern in isolation, and the directional movement results from these CPGs oscillating at a coordinated temporal order (Cohen and Wallen, 1980; Grillner, 1985). Specifically, the sequential activation of CPGs from head to tail drives forward movement, and the reversal of their temporal order drives the backward movement.

How do animals determine the timing and order of these segmental CPGs, and reorganize them to allow smooth transition between forward and backward movement? In several animal models, the choice of movement directionality appears to rely on descending signals that modify the order, phase and amplitude of motor CPGs (Bidaye et al., 2014; Crisp and Mesce, 2004; Mu and Ritzmann, 2008; Rosenbaum et al., 2010). In lampreys, the direction of swimming was transmitted by descending signals from a group of command neurons called RS in the supraspinal region (Zelenin, 2011). Some RS neurons exhibited directionality-correlated activity pattern, while others were constitutively active regardless of the direction of swimming. RS neurons that displayed
different activity levels between forward and backward swimming were proposed to impose inhibitory signals onto the anterior spinal cord CPGs, which facilitated the reversal of temporal order of the CPG modules.

1.1.4.2. Walking

Many vertebrates use limbs for walking, running and galloping; each of these motor activities involves a different form and characteristics of the limb movement (Grillner, 2006; Kiehn, 2006, 2011). In this section I will focus on walking, which involves at least two layers of orchestrated muscle activities: the contralateral inhibition between the left and right limbs, and the flexor and extensor muscle alternation in each limb (Kiehn, 2011).

In limbed animals, establishing the left-right alternation requires interneurons called the commissural interneurons (CINs). CINs project axons cross the body midline and mediate contralateral inhibition between the left and right motor CPGs. On each side, CINs receive excitatory synaptic inputs from the motor CPG on the ipsilateral side, and make inhibitory synaptic outputs to motor neurons on the contralateral side (Kiehn 2011; Fig 1-3a). In rodents, CINs establish contralateral inhibition through a dual inhibition system, via two classes of CINs: 1) the inhibitory class, CINi, that projects directly to the contralateral motor neurons and inhibits their activities, and 2) the excitatory (glutamatergic) class, CINE, that synapse onto a class of inhibitory interneurons on the contralateral side, which subsequently make inhibitory inputs onto the same motor neuron group (Kiehn, 2011; Quinlan and Kiehn, 2007). A third class of CINs make excitatory output to the contralateral motor neurons; they were postulated to promote
synchronous left-right CPG activity, which is required to generate other forms of movements such as hopping and galloping (Kiehn, 2011).

In both left and right limbs, CPG patterns that control flexors and extensors are anti-phasic to enable movements around the limb joints. The ipsilateral inhibitory interneurons play a role in establishing such a phasic relationship (Jankowska, 2001; Kiehn, 2011). There are at least two classes of inhibitory interneurons: 1) the Ia interneurons. They receive synaptic inputs from motor neurons that innervate either flexors or extensors, and send inhibitory output to extensors or flexors respectively. This way, upon activated by the motor neurons that innervate flexors or extensors, they inhibit the contraction of antagonizing muscle group (Jankowska, 2001; Kiehn, 2011; Windhorst, 2007). 2) The second class of inhibitory neurons, the Renshaw cells receive synaptic inputs from motor neurons in flexors or extensors, and project onto the same motor neuron group. The inhibition of motor neuron by Renshaw cell allows the decrease of motor neuron and muscle activities (for example, the flexor), coinciding with the increase of activity in the antagonizing muscle group (extensor). Both Ia and Renshaw cells partake in the establishment of the flexor-extensor alternation (Fig 1-3b) (Jankowska, 2001; Windhorst, 2007).

Additional inhibitory interneurons, such as a group of GABAergic interneurons that reside in the dorsal spinal cord, likely contribute to the establishment of the flexor-extensor alternation (Wilson et al., 2010). Due to functional redundancy, it remains a challenge to identify all components that contribute to the establishment of rhythmicity and phase relationship between the flexor and extensor units, particularly in the scope of
adaptive gait and the switch between different modes of limb movement that constitute walking, running, hopping and galloping.

In summary, studies of multiple motor circuits demonstrated a conserved mechanism that underlies movement, where CPGs that reside in limbs or body segments drive rhythmic motor behaviors via intrinsic oscillation, while upper layer interneurons and proprioceptive sensory feedbacks orchestrate and fine-tune the phase and rhythm of CPGs, rendering both flexibility and adaptively of motor output under various environmental conditions.
Figure 1-3

A  Left  Contralateral Inhibition  Right

CINI  INi  MN

CPG network

CINei  INi

Synchrony

B

Flexor Muscle

Flexor MN  CPG network

RC  la

Extensor Muscle

Extensor MN  CPG network

RC
Figure 1-3. A diagram for a dual mechanism model that underlies left-right alternation and flexor-extensor alternation in the rodent limbs

A. Proposed organization of circuit connection that underlies left–right coordination. The core of the contralateral inhibition is mediated by CINs (commissural interneurons), which act both directly by the inhibitory CINs (CINi), and indirectly by the excitatory CINs (CINEi) on the contralateral motor neurons (MNs). The rhythm generating CPG network activates CINi and CINEi. The CINi send axons crossing the midline to innervate inhibitory interneurons (INi) on the contralateral sides. The INs in turn inhibit ipsilateral motor neurons and rhythm generating CPG network neurons. Such a mechanism led to left-right alternation. Left–right synchrony is achieved through engaging a single excitatory system (CINE) that activate directly on the contralateral motor neurons.

B. A diagram of proposed mechanisms for the flexor and extensor alteration CPGs. Flexor and extensor motor neurons are driven to rhythmicity by alternating excitation and inhibition. Candidate premotor inhibitory neurons are the Ia interneurons that are wired in a reciprocal pattern. The CPG network activating flexor motor neuron also sends output to the Ia that inhibit extensor motor neurons. Similarly, the CPG that excites extensor motor neurons activates the Ia to inhibit flexor motor neurons. Renshaw cells (RCs) are another groups of inhibitory interneurons that are activated by motor neurons, which reciprocally inhibit the motor neuron to decrease their activity. These two mechanisms are postulated to secure the flexor–extensor alternation of the same limb.

INi: ipsilaterally projecting inhibitory interneuron. MN: motor neuron. Excitatory and inhibitory interneurons are colored coded orange and blue, respectively. Arrowheads and horizontal lines indicate excitatory and inhibitory output, respectively. The midline is denoted by the dotted line. Adapted from Kiehn, 2011.
1.1.5. Multi-functional circuits

An extreme case of motor circuit flexibility is multi-functionality, where a motor circuit is capable of generating multiple activity patterns using a similar set of cellular components. The versatility of multifunctional circuits relies on the capacity of CPG neurons to fire with different strength and pattern subjected to neuromodulation and sensory feedback (Marder, 1994).

There are two modes of activity pattern switching for multi-functional motor circuits: a graded change of rhythm (such as gait and velocity modulation), and a discrete change between two distinct patterns (such as walking and galloping). First described by Getting (1989), the neural network in the sea slug Tritonia exhibits two distinct escape behaviors. In the presence of chemical cues, Tritonia executes an avoidance response in forms of swimming, whereas a tactile stimulus triggers a defensive withdrawal. During the examination of the animal’s isolated brain preparation, it was found that the same interneuron network generated different neural activity patterns for swimming or withdraw, depending on the level of sensory inputs (Getting, 1989; Getting and Dekin, 1985).

Briggman and Kristan (2008) coined the term “multifunctional circuitry” to emphasize the adaptive property of the neural network that can drive multiple behaviors to optimally benefit an animal’s survival in a given environmental setting. The multifunctionality of motor circuits has been observed in both vertebrates and invertebrates, and is proposed as to not only increase the versatility of neural networks, but also to counteract damages of the nervous system during evolution (Briggman and Kristan,
1.2. Neural circuits undergo anatomical and functional changes during development

In most organisms, neural circuits increase in numeric and morphological complexity for neurons, support cells, and their connections during development. It is logical that a developing circuit also modifies properties in order to sustain its functional output to accommodate development, as well as to provide a layout for the mature circuit.

In many species, as early as in embryos where movement is highly restrained, the developing motor circuit has already begun to exhibit CPG-like activity and primitive motor outputs (Hanson and Landmesser, 2003; Hanson et al., 2008; Richards et al., 1999; Suster and Bate, 2002). During the course of larval or postnatal development, more descending control and ascending feedback are incorporated into the existing and functional circuit, as the system maintains its output with increased precision and versatility. To date, however, we have only limited understanding on how a circuit optimizes its activity patterns during its anatomic maturation.

1.2.1. Dynamic and systemic property modification during early development

1.2.1.1. Spontaneous motor activity promotes neural development

Spontaneous and rhythmic neural activity has become a characteristic feature of the developing nervous system, including the neonatal spinal cord and brainstem of rodents...
and chicks, and the Drosophila embryonic motor neurons (Hanson and Landmesser, 2003; Hanson et al., 2008; Ren and Greer, 2003; Suster and Bate, 2002). Accumulating evidence on early motor activities across species raised an intriguing hypothesis, that the spontaneous activity plays an important role in early development, such as cell migration, neurite growth and guidance, and synapses formation.

**Cell migration**

Rhythmic excitations, in particular those associated with rhythmic calcium influx, regulates neural migration (Komuro and Rakic, 1992, 1993, 1996). For example, the mouse cerebellar granule neurons exhibit a positive correlation between cell motility and its calcium level, largely mediated by the N-type and NMDA-type calcium channels (Komuro and Rakic, 1993, 1996).

**Axon growth and guidance**

In the chick embryonic spinal cord, spontaneous glycinergic excitatory motor neuron activities promote expression of both guidance and adhesion molecules, facilitating motor axon path-finding (Hanson et al., 2008).

**Synaptic formation and refinement**

Spontaneous neural activity has been shown to be critical for establishing neuronal connection, particularly for pre- and post-synaptic coupling at the neuromuscular junctions. In developing mouse motor neurons, spontaneous rhythmic discharges coincide with the formation of NMJs and motor activity (Marder and Rehm, 2005; O'Donovan, 1999). It was also demonstrated that the spontaneous motor neuron activity ensures pre- and post-synaptic component interaction at the developing NMJs (Reviewed by Moody and Bosma, 2005).
Spontaneous activity also promotes the expression of adhesion molecules that regulate synapse formation. In cultured mouse dorsal root ganglion neurons, electrical stimulation at specific frequencies could affect the expression of different types of cell adhesion molecules (Itoh et al., 1997; Watt et al., 2000). Such a change may further modify the growth cone of spinal neurons to recruit or remove receptors for specific neurotransmitters to promote or prevent synapse formation (Moody and Bosma, 2005).

Intrinsic neural bursting is also critical for axon pruning and synaptic refinement. During retinal development, spontaneous neuronal activity is required for the pruning of visual projections at multiple target sites, including the superior colliculus and the cortex (Torborg and Feller, 2005).

1.2.1.2. Properties of synaptic transmission channels during early development

Ion channels in developing neurons can play drastically different function from those in mature neurons. This is a combined effect of differences in their intrinsic properties (e.g. subunit composition), expression level, as well as the intracellular and extracellular ionic compositions (Reviewed in (Moody and Bosma, 2005)). In developing neurons, the constituents and electrical properties of ion channels are optimized for generating activity patterns that regulate development, such as the spontaneous activity observed in developing motor neurons and retina, instead of optimizing for mature circuit functions. In the following section I focus on developmental changes for ligand-gated ion channels and their functional implication.

Intrinsic properties of the acetylcholine receptors
Many functional differences in ion channels result from a change in their intrinsic property during development. One example is the acetylcholine receptor (AChR), which has different subunit composition between immature and mature NMJs. The fetal AChR complex contains a $\gamma$ subunit that permits a longer channel opening (Fischbach and Schuetze, 1980). This property was believed to compensate for the lower membrane conductance of the fetal muscle, providing functional innervation required for muscle maturation (Moody and Bosma, 2005). After NMJ formation, motor neuron innervation initiates the replacement of the fetal AChR with a complex that includes the $\varepsilon$ instead of $\gamma$ subunit (Mishina et al., 1986; Missias et al., 1996). This results in a reduced channel opening time, coinciding with an altered membrane property of mature muscles (Missias et al., 1996).

**Physiological nature of GABAergic synaptic transmission**

A change in the physiological nature of GABA signaling marks perhaps the most drastic development change between immature and mature nervous system documented to date. GABA is a well-studied inhibitory neurotransmitter in mature nervous system. It was first recognized in the neonatal rat hippocampal slices that GABAergic synaptic transmission is excitatory, instead of inhibitory in many regions of the developing brain (Ben-Ari et al., 1989) (Ben-Ari, 2002; Dammerman et al., 2000; Ganguly et al., 2001; Leinekugel et al., 1995). Subsequent studies showed that in neonatal brains, GABA-mediated membrane depolarization was not caused by a difference in the ion conductance or other properties of embryonic GABA$_A$ receptors, but rather, by a difference in the intracellular/extracellular chloride gradient (Staley and Smith, 2001). In an immature CNS, the intracellular Cl$^-$ concentration is much higher than that of the mature nervous
system. Therefore, the opening of GABA\textsubscript{A} receptors, a ligand-gated Cl\textsuperscript{-} channel, allows for the efflux, instead of influx of Cl\textsuperscript{-}, resulting membrane depolarization (Fukuda et al., 1998).

The intracellular chloride concentration is established mainly by two ion pumps, the Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{-} and K\textsuperscript{+}-Cl\textsuperscript{-} co-transporters, both highly expressed at early developmental stages (Delpire, 2000; Fukuda et al., 1998; Rohrbough and Spitzer, 1996). The onset of inhibitory GABA signaling coincides with the upregulated expression of another K\textsuperscript{+}-Cl\textsuperscript{-} - coupled-co-transporters, KCC2. Expressed predominately in mature neurons, KCC2 excludes chloride from neurons, reversing the extracellular-intracellular chloride gradient (Fukuda et al., 1998; Rivera et al., 1999). Therefore, in mature neurons, the activation of GABA\textsubscript{A} receptors leads to an influx of chloride and subsequently, membrane hyperpolarization (Rivera et al, 1999).

During early development, excitatory GABA signaling is believed to play a trophic role on nervous system development. Such an effect is likely mediated through a synergistic effect between GABA and NMDA receptors. Chloride-driven membrane depolarization induced by the opening of GABA receptors promotes the removal of the magnesium block of NMDA receptors, initiating large calcium influx. Intracellular calcium rise regulates several signaling pathways, including BDNF-regulated gene transcription (Berninger et al., 1995; Obrietan et al., 2002).

1.2.1.3. Coordinating maturation of developing neural circuits

As described earlier, extensive studies have been carried out to address the cellular maturation during development. At a system level, as more cellular elements are
embodied into the neural networks, circuit activity should adapt to these anatomic changes to maintain or alter its behavioral output.

Only a few studies describe the developmental changes of neural circuit. One of the early studies by Sillar and colleagues examined the swimming behavior in the developing *Xenopus laevis* larvae (Sillar et al., 1991). Upon hatching, *Xenopus* embryos were capable of a stereotypic, rigid and rhythmic swimming, before the formation of descending modulatory connection and of peripheral feedback to the spinal cord motor neurons. After a brief post-hatching period, as the connections form between interneurons and spinal cord CPGs, a remarkable increase in the flexibility of swimming rhythm was observed (Sillar et al., 1992; Sillar et al., 1991). These behavioral changes coincided with the spinal cord neurons acquiring the capability to generate burst patterns of flexible duration, rendering a refinement muscle contraction control. The development of descending projection of serotonergic interneuron was found to be necessary for the bursting spinal cord neurons to generate adaptive fictive swimming patterns (Sillar and Roberts, 1993). Therefore, the output of motor circuit is optimized as post-embryonic modification of the motor circuit wiring takes place anatomically.

Until recently, addressing how overall circuit activity patterns evolve has remained technically impossible due to a lack of non-invasive methods to reveal network activity in intact and behaving animals. The astronomical complexity of vertebrate CNS organization adds another layer of complexity. In this regard, small invertebrates offer an accessible experimental model, aided by a simple and much better understood architecture of the nervous system, accessibility for electrical and optical physiology analyses, and quantifiable behavioral output in large populations. *C. elegans* is one such
model organism that offers several advantages for systems level analysis on neural circuit maturation.

1.3. **The *C. elegans* model for motor circuit development and function**

1.3.1. **C. elegans as a model to study neural network function**

*C. elegans* is a small, fast-growing, and free-living invertebrate that is easily cultivated at large quantity in the laboratory. Using a simple nervous system that consists only 220 neurons at birth, and 302 neurons in adults, *C. elegans* generates a rich repertoire of quantifiable locomotory behaviors that constitute three basic motor modules: run, turn and reversal. Responding to a variety of sensory stimuli, *C. elegans* generate navigation, dwell, avoidance or escape motor behaviors that are assembled from these modules. In addition to locomotion, *C. elegans* exhibit other forms of motor activities including feeding, defecation and egg-laying (Brenner 1976, white et al., 1986).

*C elegans* offers many advantages for developmental studies, including a near complete reconstruction of the adult nervous system at synapse resolution (Chen et al., 2006; White et al., 1976), a fully sequence genome (*C. elegans* sequencing consortium, 1998), a large collection of genetic mutations (Brenner, 1974), and a complete cell lineage map from fertilization to adulthood (Sulston, 1983). The optical transparency of the animal, technical advancement in imaging, optogenetics, and neurophysiology has allowed the field to fully capitalize the strength of *C elegans* as an experimental system to dissect circuit development and function across the molecular, cellular and systems level, at single neuron, up to single synapse resolution.
Several powerful tools to dissect circuit function have been particularly well adapted and optimized for *C. elegans* studies. For example, cell ablation by micro-beam laser has provided the crucial insight on circuit components for navigation (Gray et al., 2005), touch (Chalfie et al., 1985), tap and escape responses (Gray et al., 2005; Wicks et al., 1996). Optogenetic ablation, where acute cell death is induced by exogenously expressed proteins that generate oxidative stress upon light stimulation, now allows for multi-neuron ablation in a population, at any given developmental stage (Chalfie et al., 1985; Kobayashi et al., 2013; Qi et al., 2012). Reversible manipulation of neuronal activity (activation and inactivation) in intact and behaving animals has become possible with light- or small molecule or compound-gated ion channels and ion pumps. These tools provide means to correlate neuronal activity to specific behavioral outputs (Donnelly et al., 2013; Faumont et al., 2011; Liewald et al., 2008; Nagel et al., 2005). Live imaging of neuronal activity using genetic calcium or voltage sensors provides a real-time measurement of the activity patterns of the neural network in behaving animals (Ben Arous et al., 2010; Faumont et al., 2011; Kawano et al., 2011; Kerr et al., 2000; Tian et al., 2009; Wen et al., 2012). The combinatory application of neurophysiology techniques, such as direct measurement of the downstream neuron activity upon stimulation or inhibition of targeted neurons, promises dissection of the neural network activity with finer resolution.

### 1.3.2. Anatomic connectivity of the mature *C. elegans* motor circuit

A reconstruction of the wiring diagram of the *C. elegans* nervous system by John White and colleagues (1976) led to the identification of candidate sensory neurons, motor
neurons and premotor (command) interneurons. Together with the laser ablation studies on some of these neurons, these studies defined the major units in the adult *C. elegans* sensorimotor circuit (Chalfie et al., 1985; White et al., 1976, 1986). In a basic framework, sensory neurons gather tactile and ambient stimuli from the environment, and make chemical or electrical synapses to layers of interneurons that are further dived into two subclasses: the primary interneurons that receive direct inputs from sensory neurons, whereas the secondary interneurons that mainly make synaptic contacts with other interneurons (White et al., 1986). These sensory inputs influence the selection of motor programs and modulate motor circuit activity through synaptic communications with primary or secondary interneurons that in turn communicate with the premotor interneurons. Mechanosensory inputs can also directly communicate with premotor interneurons and motor neurons through monoamine release and monoaminergic signaling (Donnelly et al., 2013).

The core elements of the *C. elegans* adult motor circuit consist a small number of premotor interneurons and several classes of motor neurons. Five pairs of premotor interneurons, previously known as the command interneurons, AVB, PVC and AVA, AVD, AVE, integrate inputs from the sensory and upper layer interneurons, and provide the majority of synaptic output to musculatures. They are extensively interconnected by chemical and electrical synapses (White et al., 1976, 1986). These premotor interneurons reside in the head (AVB, AVA, AVD and AVE) or tail (PVC), and extend axons along the entire length of the ventral nerve cord (VNC), where they form both electrical and chemical synapses with motor neurons that are distributed along the VNC and innervate body wall muscles (White et al., 1976, 1986; Fig. 2-1A).
There are 5 classes of VNC motor neurons: A, B, D, AS and VC. Each class innervates the musculature of a small body segment (White et al., 1976, 1986). Three classes of motor neurons, the A, B and D, make the majority of NMJs to body wall muscles. The AS and VC motor neurons contribute only a small fraction of NMJs to the dorsal and ventral body wall muscles, respectively (White et al., 1976). The A, B and D motor neurons are further divided into the dorsal and ventral subclasses, which innervate the dorsal (DA, DB, DD), or ventral (VA, VB, VD) body wall muscles, respectively (White et al., 1976, 1986). Both A and B motor neurons are excitatory cholinergic motor neurons (Duerr et al., 2008; Richmond, 2009), with A motor neurons projecting anteriorly-directed axons, and B posteriorly-directed axons. D motor neurons project both axons and dendrites anteriorly to their soma, which reside on opposite sides of the dorsal/ventral axis, and are connected by circumferential commissures ((White et al., 1986); Fig 2-1 A). They are GABAergic and inhibitory to muscle contraction (Chalfie et al., 1985; McIntire et al., 1993).

The adult VNC is predicted to contain six repeating modules of about 12 motor neurons consisted of A/AS, B and D, and 12 muscle cells, based on a few motifs analyzed with the EM reconstruction (Haspel and O'Donovan, 2011, 2012; White et al., 1976). These repeated A/AS, B and D modules receive synaptic inputs from premotor interneurons: A/AS form gap junctions and chemical synapses with premotor interneurons AVA, AVD and AVE, whereas B form electrical and chemical synapses with AVB and PVC, respectively. D do not receive synaptic inputs from premotor interneurons; instead, they are postsynaptic to A and B in the same module, and
presynaptic to body wall muscles opposite to those innervated by A and B (Chalfie and White, 1988; White et al., 1976, 1986).

1.3.3. Circuit mechanisms underlying sinusoidal movement in adult *C. elegans*

1.3.3.1. The locomotion pattern of laboratory *C. elegans* strains

*C. elegans* exhibits two different mode of locomotion, depending on the media of navigation: crawling in soil and swimming in liquids. Wild *C. elegans* likely switch between these modes in response to different friction and viscosity of its natural habitat.

In the laboratory, *C. elegans* is typically cultivated on agar-based NGM plates. They lie on laterally, crawl with rhythmic and propulsive undulation - a propagating bending wave consisted of phase-coupled contraction and relaxation of ventral and dorsal body wall musculature. The amplitude and frequency of undulation, and directionality of sinusoidal motion are dynamically regulated by the sensory and motor circuits, which allow the movement pattern to adapt to its environment (Ben Arous et al., 2010; Croll, 1975; Fang-Yen et al., 2010; Pierce-Shimomura et al., 2008).

The bending wave propagation from the head to tail results in forward locomotion, while the propagation in a tail-to-head direction results in reversal. In the absence of food and other sensory stimuli, *C. elegans* exhibits forward bias in locomotion (Gray et al., 2005). In the presence of sensory cues, animals respond with a change in frequency of reversal and turns, resulting in taxis or avoidance behaviors (Bargmann and Mori, 1997). Many studies have demonstrated that directional movement is tightly coupled with the activity level of components of the motor circuits, modulated by sensory feedbacks (Chalfie et al., 1985; Gray et al., 2005; Hills et al., 2004; Sawin et al., 2000).
1.3.3.2. Mechanisms underlying undulation

The anatomic connectivity of the *C. elegans* motor circuit predicts a reciprocal inhibition model for undulation. Specifically, analogous to the model proposed for the nematode *Ascaris* (Walrond and Stretton, 1985; White et al., 1986), when the body wall muscles contract on one side, muscles on the opposing side must relax to allow bending.

The A/AS, B and D motor neuron modules were postulated to coordinate the cycle of dorsal/ventral muscle contraction and relaxation to generate sinusoidal motion (White et al., 1986); Fig. 1-4). The cholinergic A and B motor neurons form dyadic synapses to muscles and D motor neurons; this postulates a functional model that A and B activation can simultaneously lead to muscle contraction, and induce contralateral muscle relaxation through D (Gao and Zhen, 2011; Liewald et al., 2008; White et al., 1986). For instance, while the DA or DB motor neurons activate the dorsal muscles, the simultaneous activation of VD motor neurons will signal the ventral muscles of the same body segment to relax. Here D motor neurons act as the reciprocal inhibitors.

Several studies lend support to this reciprocal inhibition model. The selective elimination of DD or VD motor neurons by optogenetic ablation resulted in biased bending towards dorsal or ventral side during navigation, respectively. Consistently, ontogenetic stimulation of DD alone induced ventral biased bending during navigation, possibly from prolonged dorsal muscle relaxation (Donnelly et al., 2013). Lastly, a genetic mutant that lacks GABA signaling, *unc-25/GAD*, which cannot synthesize GABA, shrink when stimulated to generate fast forward movement, a behavior likely caused by simultaneous muscle contraction of the entire body (McIntire et al., 1993).
The same studies however raise questions on the necessity of reciprocal inhibition for undulation. Dorsal and ventral bending was not abolished upon the ablation DD or VD motor neurons, respectively (Donnelly et al., 2013). When not stimulated, *unc-25* mutants maintain undulation and sinusoidal waves during forward locomotion, albeit the movement pattern being slower and animals being slightly hypercontracted (McIntire et al., 1993). These findings imply that other mechanisms, possibly in parallel to DD-mediated reciprocal inhibition, facilitate alternating dorsal and ventral muscle contraction during undulation.

### 1.3.3.3. Dedicated circuits for directional movement

The directional propagation of body bending enables directional movements: an anterior to posterior propagation drives forward movement, whereas a posterior to anterior propagation promotes backing. Work from several groups has begun to provide clues on the circuit mechanism that underlies directional movements.

**Premotor interneurons**

Directional movement in *C. elegans* is modulated by two functionally separated networks in the motor circuit (Chalfie et al., 1985; Kawano et al., 2011; Wicks et al., 1996). Simultaneous laser killing of AVB and PVC abolished the animals’ ability to move forward in response to the tail touch, whereas ablating AVA and AVD rendered animals unable to move backward in response to the head touch (Chalfie et al., 1985; Wicks et al., 1996). Hence, premotor interneurons have biased functional contribution for directionality. Specifically, AVB and PVC are primarily associated with promoting forward movement, while AVA and AVD with reversal. The ablation of individual
interneurons caused different level of impairment in locomotion: the ablation of AVB or AVA interneurons caused more severe impairment of spontaneous forward or backward movement, respectively; the ablation of PVC or AVD dampened touching-evoked forward and reversal response while leaving unnoticeable effect on spontaneous locomotion (Chalfie et al., 1985). These studies establish AVB and AVA as the main regulators for forward and backward movement, respectively.

A few groups, including our group, developed a real-time in vivo calcium imaging system to monitor the motor circuit activity in intact, moving animals, allowing a direct correlation between the motor circuit activity and motor output (Kawano et al., 2011; Wen et al., 2012; Xie et al., 2013). We observed an in-phase increase of the AVA, AVE and RIM calcium transient that coincided with the initiation and the sustaining of backward movement. In contrast, an increased calcium profile in AVB correlated with forward movement. A remarkable anti-correlation between AVE and AVB was observed in animals fairly restricted for locomotion, indicating an intrinsic cross-inhibition between the forward and reversal-driving premotor interneuron networks (Kawano et al., 2011).

**Motor neurons**

The B and A/AS motor neurons are the main post-synaptic partner of the AVB and PVC, and the AVA, AVE and AVD premotor interneuron group, respectively (White et al., 1976). Do they function in the sub-circuit in line with their presynaptic partners?

The first attempt to address the role of different motor neuron groups was also made through laser ablation. These studies were however were less informative due to the large number of motor neurons, and massive changes in motor neurons during postembryonic
larval development (described in details in later sections). In the newly hatch larvae (L1), laser ablation of a portion of the A and B motor neurons showed distinctive behavioral alternation: killing some B impaired forward locomotion, whereas killing some A abolished reversal in L1. Killing D led to compromised forward and backward movement (Chalfie et al., 1985). However, the effect of complete ablation of the A/AS, B and D motor neuron population in L1 remains unknown.

Kawano et al. (2011) co-imaged the B and A motor neuron activity in moving animal using a FRET-based calcium sensor cameleon. VB9 and VA8, the B and A motor neurons that make synaptic inputs to the same muscles, were imaged during episodes of active shuttling forward and backward movement. A differential level of calcium activity between VB and VA that correlates with directional movement and their switch was observed: VB became more active the VA during forward movements, and vice versa during reversal.

Taken together, these results suggest that the AVB and PVC premotor interneurons and their main postsynaptic partners, the B motor neurons promote forward movement. On the other hand, the AVA, AVE and AVD premotor interneurons, through innervating the A and AS motor neurons, promote backing. Through a combination of genetic and optogenetic studies, we have shown that the activity difference of the forward and backward circuit output modules, A and B, not only correlates with, but is also necessary and determines the directionality of movements (Kawano et al., 2011).

1.3.3.4. Reciprocal activation of two motor neuron pools
The functional separation of key motor circuit components, at both premotor interneuron and the motor neuron level, predicts the necessity of intrinsic mechanisms to prevent their simultaneous activation. A cross-inhibition pattern was indeed observed in the premotor interneurons: AVA/AVE and AVB exhibited a significant anti-correlated activity profile during directional locomotion (Kawano et al., 2011). Consistently, VB and VA motor neurons maintain a difference in their calcium level; and critically, a simultaneous and reciprocal change of VA and VB calcium transient coincided with the transition between directional movements. Cumulatively, these results indicate that an imbalanced output of the two motor sub-circuits underlies the directionality of C. elegans movement. The reciprocal activation of the interneurons, AVB and AVA/AVE, facilitates the establishment and shift between activity difference between B and A/AS motor neurons (Kawano et al., 2011).

1.3.4. The larval motor circuit

The functional model of the C. elegans motor circuit is based on the wiring and activity pattern of the adult nervous system. However, the C. elegans motor circuit undergoes continuous postembryonic development, increasing the motor neuron number from 22 by birth to 75 by the end of the larval development. With a defined cell lineage, the framework for the developmental modification of the C. elegans motor circuit has been precisely described. A newly hatched L1 C. elegans possesses only 22 motor neurons, consisted of the DA, DB and DD subclass of the A, B and D motor neurons, in the VNC (Sulston and Horvitz, 1977; White et al., 1978; Fig 2-1B). The vast majority of postembryonic neural developmental events takes place in the second-half of L1 stage: 54
new motor neurons, within a period of ~6 hours, are incorporated into the VNC; they include all VD and AS motor neurons, and all except the one VA and VB motor neurons that constitute the adult motor circuit (Sulston and Horvitz, 1977). Hence, the L1 larvae have very different anatomic structure from the rest of larvae and adults. Below I describe what is known about the motor neurons of the L1 stage larvae.

**L1 DA and DB motor neurons**

In L1 larvae, only the DA and DB subclass of the A and B motor neurons are present. The connectivity and property of the L1 DA and DB motor neurons remain largely unknown, but they are likely to be excitatory, as in adults, as implicated by results from the laser ablation studies (Chalfie et al., 1985). In the L1 larvae, ablation of several DA motor neurons left the animal severally impaired the larvae’s ability to move forward, whereas the ablation of a few DB severely disrupted forward locomotion (Chalfie et al., 1985). This suggested that DB and DA motor neurons could similarly participate in promoting forward and backward locomotion by innervating muscle contractions. Specific mechanisms for how they are organized to underlie locomotion remains to be explored. In the following chapter, I present unpublished studies that provide supporting evidence that the L1 DA and DB motor neurons activate muscle contraction.

**L1 DD motor neurons**

Similar to the case of the A and B motor neurons, only DD subclass of the D motor neurons is present at newly hatched L1 larvae. Laser ablation of DD neurons in L1 led to disruption of both forward and backward locomotion, implying their participation in circuits that potentiate both forward and backward movement (Chalfie et al., 1985). The L1 DD neurons are GABAergic, as they express GAD (UNC-25). However, it was noted
that the L1 DD neurons exhibit a reversed pre- and post-synaptic polarity compared to DDs in adults - they are postsynaptic to DA and DB neurons on the dorsal side, and presynaptic to ventral muscles (Sulston and Horvitz, 1977; White et al., 1978).

Several studies have begun to reveal intriguing characteristics of a post-embryonic developmental remodeling event by the DD motor neurons. In early L1 larvae, the embryonically born DDs form NMJs with ventral muscle, while receiving presynaptic input from the DA and DB motor neurons on the dorsal nerve cord (DNC). Using presynaptic markers specifically expressed in the DD motor neurons, it was shown that within the last 3-5 hour of the L1 stage, DD axons began to remove NMJs to dorsal body wall muscles, and to form dorsal muscles in an anterior to posterior order (Hallam and Jin, 1998; Walthall et al., 1993; White et al., 1978). The completion of this process was described as the beginning (Hallam and Jin, 1998) or the end of the second larval (L2) stage (Kurup et al., 2015).

In addition to wiring change, it remains unknown whether the nature of L1 GABA signaling undergoes the excitatory to inhibitory change as described in the mammalian nervous systems. In many vertebrates, in immature neurons or developing circuits, GABAergic synapses are excitatory due to a reversed intracellular chloride level (Fukuda et al., 1998; Rivera et al., 1999). A recent study found that an acute ablation of all L1 DD neurons led to biased ventral body coil in liquid (Williams et al., 2013), which is consistent with DD exhibiting an inhibitory role on muscle contraction. Consistently, another study reported that despite the up-regulation of the C. elegans KCC2 in the mid-L1 larvae, DD motor neurons likely remain inhibitory to body contraction (Han et al., 2015).
If the partial described L1 motor circuit is correct, and the DA and DB provide excitatory innervation on dorsal muscles, and DD are inhibitory to ventral muscles, we are left with an asymmetric motor system – with only excitation and inhibitory input to the dorsal and ventral muscle, respectively. Yet, *C. elegans* maintain similar movement pattern with similar biophysical properties upon hatching and throughout development (Backholm et al., 2013). This elicits an unresolved question on how L1s generate movements.

1.3.5. **Unresolved questions regarding the developing L1 *C. elegans* motor circuit**

A fundamental question to be addressed is how the L1 stage larvae produce undulation with the profound ventral/dorsal excitatory and inhibitory input asymmetry as implied by the predicted L1 wiring diagram? This question may be further divided into specific problems: Firstly, are the L1 motor neuron activities organized as predicted by the asymmetric model during movement? Second, if so, whether muscle activity reflect or overcome the asymmetric neuronal input? In the next chapter, I describe results where I addressed these questions using three different approaches:

1) I examined the activity patterns of motor neurons during locomotion during early L1 stage. I performed analyses to examine the correlation of different classes of cholinergic A and B motor neurons with forward and backward locomotion, and the correlation of GABAergic D motor neurons with muscle contraction and relaxation during both forward and backward movement.

2) I examined whether D motor neurons facilitate reciprocal inhibition. Albeit the proposed role to relax muscles opposing to those that are contracted by cholinergic motor
neurons, a demonstration of such a mechanism however has never been experimentally examined. Since the animals preserve the sinusoidal movement prior and post to the synaptic remodeling of DD, I further addressed whether D activity patterns undergo changes to maintain sinusoidal movement.

3) I further investigated mechanisms through which motor neuron activity controls muscle contraction and relaxation in L1 larvae. I focused on identifying the motor neurons that are associated with ventral muscle contraction and relaxation. A key puzzle piece is how the juvenile motor circuit is functionally arranged to generate rhythmic alternation of ventral and dorsal muscle contraction.

1.3.6. A summary of my thesis studies

In the next chapter, I describe the functional dissertation of the L1 *C. elegans* motor circuit. An ultimate goal is to build a functional model explaining how the L1 motor circuit governs rhythmic locomotion that coincides with its postembryonic development.

First, I investigated the functional contribution of the three classes of L1 motor neurons to locomotion by examining the behavioral effect upon optogenetic ablation of individual motor neuron classes. Through this study, we identified the motor module for directional movement. Second, my colleagues and I examined the activity pattern of L1 motor neurons and body wall muscles by calcium imaging. Through this study, I established the correlation of their activity with different modes of muscle contraction and locomotion. This chapter consists of unpublished data generated by myself, and by my colleagues in the Zhen lab.
2. The anatomic connectome and functional dissection of the *C. elegans* first-stage larvae (L1) motor circuit

2.1. Abstract

During development, a maturing neural circuit exhibits increased complexity of anatomic composition and connectivity, while maintaining or changing the behavioral output that is optimized for different developmental stages (Clarac et al., 2004; Kiehn, 2011; Ren and Greer, 2003). To date, how neural circuit activity patterns change during the anatomic and functional maturation remains a poorly described and understood biological process.

Across all developmental stages, the nematode *C. elegans* navigates through its environment by propagating sinusoidal bending waves along its body (Backholm et al., 2013; Brenner, 1974; Croll, 1975; White et al., 1976, 1986). The neural circuit controlling motor behaviors, however, is predicted to undergo major reconstruction at the end of the first larval (L1) stage. In late larvae and adults, where motor behaviors are examined in most reported studies, the ventral and dorsal muscles are symmetrically innervated by cholinergic and GABAergic motor neurons (Chalfie et al., 1985; Sulston and Horvitz, 1977; White et al., 1976). The phasic excitation of these cholinergic motor neurons, coupled with the contralateral inhibition generated by the GABAergic motor neurons, is thought to generate and propagate undulatory waves (Kawano et al., 2011; Liewald et al., 2008; Wen et al., 2012; White et al., 1986).

However, all motor neurons that innervate the ventral body wall muscles in adults, the cholinergic VB-, VA- and the GABAergic VD-type, as well as the dorsal muscle innervating cholinergic AS-type motor neurons, are born from mid- to the end of the L1 stage (Sulston and Horvitz, 1977; White et al., 1976), yet L1 animals generate
undulation and directional movement similar to adults (Backholm et al., 2013). Since the sinusoidal bending pattern is consisted of an alternating dorsal and ventral bending pattern (Butler et al., 2015), it is difficult to envision such a pattern to be generated by a circuit with a profound ventral/dorsal asymmetry in motor neuron inputs. Hence, the L1 larvae may generate its motor output either through a motor circuit consisted of different components, and/or their wiring and property of synaptic transmission.

To address how C. elegans produces undulatory behavior in the L1 stage, we took a combined approach: we are reconstructing the L1 motor circuit by serial section electron microscopy; in parallel, we applied targeted neuronal ablation, calcium imaging, and optogenetic stimulation to pinpoint the mechanism for ventral and dorsal muscle innervation. The work reported here mainly focuses on the second aspect – the functional assessment of the L1 motor circuit. Specifically, first, we investigated the functional contribution of L1 stage motor neurons to directional movement, by examining the motor pattern change upon targeted ablation of specific motor neuron groups. Next, we determined the activity pattern of each motor neuron group to establish their correlation with directional movement in the L1 larvae. Lastly, we directly assess the effect of ablating individual motor neuron groups on the muscle activity pattern by real-time calcium imaging.

**Results from above experiments led to following conclusions:**

1) The L1 motor neurons, DAs, DBs and DDs, exhibit following similar properties as in the adult circuit: **the DB and DA motor neurons are excitatory and their rhythmic activities correlate with forward and backward movement**, respectively. **The DD motor neurons are inhibitory; its activation correlates with muscle relaxation during**
movement of both directions.

2) The activity of the inhibitory DD motor neurons on ventral muscles depends on the input from DA and DB motor neurons, during forward or backward movement, respectively.

3) The L1 motor neuron’s functional output exhibits following difference from the adult circuit: the activity of excitatory DA and DB motor neurons are required for both dorsal and ventral muscle contraction, and the DD motor neurons mediate ventral, instead of dorsal muscle relaxation.

These conclusions hence favor a differential synaptic wiring or input-output relationship between the motor neurons to muscles to account for L1’s sinusoidal pattern.

Based on these results, we propose four hypotheses for how the L1 motor circuit may operate to be tested in future studies

1) Excitatory DA and DB motor neurons directly innervate dorsal and ventral body wall muscles. Such a disposition predicates that they make NMJs to ventral and dorsal muscles.

2) Alternatively, DA and DB directly innervate dorsal muscles, as well as innervate ventral muscles indirectly. This disposition predicates that they make not only synaptic connections to dorsal muscles, and in addition, to unknown neurons that in turn activate ventral muscles.

3) DD motor neurons account for both dorsal and ventral relaxation. This disposition predicates that they make NMJs to both ventral and dorsal muscles.

4) Alternatively, DA and DB’s self-maintained oscillation generates periodic dorsal muscle relaxation. This predicts that DDs make NMJs to only ventral muscles.
Future experiments to distinguish these possibilities will be guided by results from our ongoing L1 motor circuit EM reconstruction. Through such a comparative analysis of the circuit basis between L1 and adult locomotion, we hope to shed light on conserved principles of behavioral adaptation during sensorimotor development.
2.2. Introduction

In both vertebrates and invertebrates, locomotion relies on interconnected neural networks that generate different activity patterns, and enable the executions of, and switches between motor outputs (Goulding, 2009; Grillner, 2003; Rossignol et al., 2006). Throughout development, a maturing neural circuit increases neuron numbers and connectivity complexity (Clarac et al., 2004; Marder and Rehm, 2005). Therefore its property is also tightly coupled with the developmental progress in order to maintain and optimize the functional output. How a circuit optimizes its activity patterns during anatomic and functional maturation remains poorly described and understood.

With a nervous system that has been mapped at synaptic resolution by electron microscopic (EM) reconstruction in adults (Chen et al., 2006; Varshney et al., 2011; White et al., 1986), and the recent advancement in optical neurophysiology techniques (Donnelly et al., 2013; Faumont et al., 2011; Leifer et al., 2011; Nagel et al., 2005), the nematode Caenorhabditis elegans (C. elegans) motor circuit offers an attractive model for detailed investigation of circuit operation and modulation (Ben Arous et al., 2010; Faumont et al., 2011; Kawano et al., 2011; Kerr et al., 2000; Tian et al., 2009; Wen et al., 2012). Consisted of 302 neurons in its mature nervous system, C. elegans demonstrate a surprisingly rich repertoire of behavior outputs in response to environmental variabilities (Donnelly et al., 2013; Fang-Yen et al., 2010; Gray et al., 2005; Pierce-Shimomura et al., 2008; Wicks et al., 1996).

The C. elegans motor circuit undergoes stereotypic, step-wise anatomic maturation at distinct larvae stages (Sulston and Horvitz, 1977). 22 motor neurons (MNs) are developed embryonically and are present upon hatching. Within a period of ~5hr
(under laboratory culture conditions at the 25C) towards end of the first larvae stage (L1), 54 new MNs are derived upon the sequential division of 12 postembryonic blast P cells. The last 2 new MNs arise within the first two hours of the second larval stage (L2). The postembryonic neurons are expected to be incorporated into the existing circuits, while some of the existing neurons undergo changes in synaptic wiring, such as embryonically derived DD may completely switch their NMJ input from ventral to dorsal muscles (Hallam and Jin, 1998; Walthall et al., 1993; White et al., 1978). Although the connectivity and synaptic organization are expected to change significantly, when and how the integration process take place completely lacks description. Surprisingly, the behavior output by means of biophysical properties and response to environment remains analogous throughout all larval stages (Backholm et al., 2013). The developmental characteristic of the C elegans motor circuit provides an excellent platform to study the underlying mechanism for the maintenance of behaviors and biomechanical properties in response to dynamic neural circuit connectivity changes.

On standard culture plates, a freely moving C. elegans exhibits rhythmic and propulsive undulation that propels them in forward and backward directions (Chalfie and White, 1988; Gray et al., 2005). The main components of an adult C. elegans motor circuit were defined by their anatomic connectomes, activity pattern, and functional contribution to motor behaviors: five pairs of premotor interneurons AVB, AVA, AVD, AVE and PVC integrate inputs from sensory and upper layer interneurons. They output onto motor neurons, whose soma and dendrite residing along the ventral nerve cord (VNC) (Chen et al., 2006; Haspel and O'Donovan, 2011, 2012; White et al., 1976). Each of these motor neurons innervates muscles of a small body segment (White et al., 1976,
Three classes of motor neurons provide the majority of synaptic inputs to body wall muscles: the cholinergic and excitatory A and B, and inhibitory D motor neurons (White et al., 1976, 1986) (Fig. 2-1A). Each class is further divided into two subclasses: the dorsal-muscle innervating subclass (DB, DA and DD), and the ventral-muscle innervating subclass (VB, VA and VD) for dorsal and ventral musculature, respectively (White et al., 1976, 1986). Based on the wiring diagram and activity patterns, two separate circuits are proposed to promote directional movement: premotor interneurons AVB and PVC drive forward locomotion via innervating B motor neuron through electrical and chemical synapses; the AVA, AVD and AVE premotor interneurons, together with their post synaptic partner A motor neurons are responsible for the backward locomotion (Chalfie et al., 1985; Kawano et al., 2011; Wicks et al., 1996). In adults, both ventral and dorsal muscles are symmetrically innervated by both cholinergic and GABAergic motor neurons, and phasic excitation coupled with contralateral inhibition is thought to be responsible for undulation and its directional propagation (Donnelly et al., 2013; Gao and Zhen, 2011; Leifer et al., 2011; White et al., 1976, 1986).

However, a newborn L1 C. elegans possesses only 22 motor neurons, all being the adult dorsal-muscle innervating motor neurons - the cholinergic DAs and DBs, as well as the GABAergic DDs (Sulston and Horvitz, 1977; White et al., 1976, 1986) (Fig. 2-1B). A partial EM reconstruction studies suggested that some DDs are postsynaptic to DAs and DBs in L1, and presynaptic to the ventral body wall muscles, opposite to their synaptic input and output patterns in adult C. elegans (Zhen lab, unpublished). This and other studies suggest that sometime between L1 and L2, DD motor neurons undergo a distinct remodeling event, such that they reverse their polarity, to become presynaptic to
and inhibit dorsal body wall muscles while their ventral processes become postsynaptic to the newly derived VA and VB motor neurons (Hallam and Jin, 1998; Walthall et al., 1993; White et al., 1978)(Fig. 2-1B). This remodeling event was speculated to coincide with the maturation of the post-embryonically derived, adult ventral muscle innervating GABAergic neurons (VDs).

The connectivity and activity of cholinergic motor neurons DBs and DAs remain largely unexplored in L1s. In the adult motor circuit, DBs and DAs provide excitatory NMJ inputs onto the dorsal muscles (Gao and Zhen, 2011; Liewald et al., 2008; Wen et al., 2012; White et al., 1976). If they remain the same wiring in L1s, the ventral musculatures would receive no excitatory inputs, since all ventral cholinergic motor neurons VA and VB are developed by the end of this stage. However, upon hatching, L1s exhibit sinusoidal locomotion pattern, with the capability to perform both forward and backward movement, similar to that in adults.

Hereby, an important question arises: how does the juvenile motor circuit in L1 generate rhythmic locomotion prior to the development of all motor circuit components? Specifically, what is the source of the excitation of the L1 ventral muscle, as well as the relaxation of the L1 dorsal muscle? In this study, I focus on determining the motor neuron and muscle activity patterns in L1 C. elegans that underlie directional locomotion and undulation. Our current studies suggest that L1s likely utilize different neuronal property and wiring to achieve adult-like sinusoidal locomotion. These studies provide insights into how behavioral output is maintained throughout neural circuit maturation.
Figure 2-1. Schematic diagram of anatomic organization and connectivity of C. elegans motor circuit
**A. Top Left:** A schematic diagram of the anatomic organization of a mature *C. elegans* motor circuit. It includes the premotor interneurons (AVA, AVE, AVB, and PVC), and the ventral nerve cord (VNC) motor neurons. The A-, B- and D-class motor neurons provide the majority of excitatory (A and B) and inhibitory (D) inputs to body wall muscles. The A motor neurons project anteriorly directed axons. B motor neurons make posteriorly directed axon projections. A and B motor neurons receive synaptic inputs from the premotor interneuron axons that extend along the VNC. VA and VB subclass of the A and B motor neurons innervate the ventral body wall muscles, whereas DA and DB subclasses innervate the dorsal body wall muscles. D motor neurons receive synaptic input from the A and B motor neurons, and innervate body wall muscles on the opposite side to their dendritic processes.

**Top right:** A simplified diagram of the summarized connectivity of the adult *C. elegans* motor circuit. Hexagons and circles represent premotor interneurons and motor neurons, respectively. Arrows indicate chemical synapses, and lines represent gap junctions. Proposed motor circuits responsible for forward and backward locomotion are coded in red and blue, respectively. AVB and PVC premotor interneuron and B motor neurons involved in forward locomotion, while AVA, AVE, and AVD premotor interneurons and A motor neurons are involved in backward locomotion. Ventral and dorsal D motor neurons are proposed to facilitate movement of both directions. Adapted from Kawano et al., 2011.

**Bottom:** D motor neurons are proposed to function as cross-inhibitors on the body wall muscles. VDs are post-synaptic to the dyadic, ventral NMJs by DA and DB, and are presynaptic to ventral body wall muscles. DDs are postsynaptic to the dyadic NMJs by VA and VB on the ventral side, and are presynaptic to dorsal body wall muscles. Adapted from White et al., 1978.

**B.** A proposed schematic diagram of the anatomic organization of an L1 *C. elegans* motor circuit. It includes premotor interneurons (AVA, AVE, AVB, and PVC) and VNC motor neurons, when only DA, DB and DD motor neurons are present.
2.3. Results

2.3.1. Functional Contribution of Cholinergic and GABAergic Motor Neurons in L1s

To determine and compare the functional contribution of three major class motor neurons, A, B and D, to locomotion between L1s and later larvae, we first selectively ablated each type of VNC motor neurons, and compared the effect on motor patterns.

L1s have only DA, DB and DD motor neurons. We focused on whether they function as analogous units to B (DB+VB), A (DA+VA) and D (DD+VD) in the last larval (L4) stage, respectively. We have chosen to compare L1s to L4s, instead of to adults, because L4s do not possess VC, another type of cholinergic motor neurons that mature at the end of the L4 stage and form NMJs with ventral body wall muscles (White et al., 1976, 1986), and adults may exhibit an additional layer of modulation of muscle activity in parallel to, or together with A, B and D motor neurons.

2.3.1.1. Experimental methods

miniSOG is a flavoprotein that generates singlet oxygen upon blue-light activation, and can effectively induce acute cell death without damaging surrounding tissues when its expression is targeted to mitochondria (Shu et al, 2011; Qi et al, 2012). I generated transgenic animals that stably express mitochondria-targeted miniSOG (tomm20-miniSOG) under the A- (Punc-4), B- (Pacr-5) and D- (Punc-25) motor neuron specific promoters. These strains were used to ablate the A, B, and D neurons at different developmental stages to assess the effect on motor pattern output. All constructs also co-express cytosolic RFP via a cistronic expression system to label the neuron that expresses miniSOG. All miniSOG strains were maintained in darkness prior to experiments.
For L1 behavioral analysis, L1 animals within 1 hour post hatching were exposed with blue LED on the NGM plate for 40 minutes, and allowed for recovery for 2 hours in dark. Their behaviors were recorded on NGM plates using a bright field microscopic setup with automated tracking system. All recordings were performed before 6 hour post-hatching. For L4 behaviors, L2/L3 transgenic animals were exposed under blue LED light for 40 minutes, and allowed recovery in darkness. Early L4 animals on a bright field microscope with automated tracking were recorded the next day. All recorded animals were visually examined afterwards to ensure the complete ablation of intended neurons. Controls for both L1 and L4 analyses were the same set of transgenic animals cultured alongside under the same manipulations except the LED exposure. Pooled data were generated from recordings using the NGM plate and recorded on the same day. These animals were transferred to a plate with thinly seeded OP50. Recording was started after 1 minutes of habituation after the animal was transferred on to recording plate and lasted for 3 minute.

For motor behavioral analysis, Taizo Kawano, a former postdoctoral fellow in the lab custom-developed a system where a single worm tracking is efficient and reliable in all developmental stages including L1s. An image J based plugin was used for post-imaging processing to extract multiple parameters for motor behaviors. This system detects the shape of the animal, divides the skeleton of the animal into 33 equally spaced segments and calculates the curvature of each segment, and extracts its central point for velocity and direction analysis. I quantified the propensity for directional movement (percentage of time animals spent in forage, reversal and pause), and separately analyzed periods of forward and backward movements on three main parameters: velocity,
duration of sustained directional movement, and frequency of re-initiation of directional movement.

2.3.1.2. Effect of B-type motor neuron ablation on L1 (DB) and L4 (DB and VB)

Upon ablating all B-class motor neurons, the forward movement of L1 and L4 larvae was similarly and severely impaired; by contrast, the backward movement of L1 and L4 larvae was more differentially affected.

a. *Forward motor patterns were altered similarly in L1 and L4 animals.*

In L1 animals, the DB ablated animals exhibited an uncoordinated undulation during forward movement: They retained a fairly normal head swing but the posterior bending propagation was drastically slower and often disrupted. L4 animals without B motor neurons exhibited changes of a similar manner, but to a more severe degree: they also retained a fairly normal head swing followed by a quickly reducing body bending propagation, which became diminished in the posterior half of the body (Fig 2-2A).

In addition to the gradually diminishing posteriorly propagating bending waves, both B ablated L1 and L4 animals exhibited a *kinker* phenotype: during the forward locomotion, animals may sometimes simultaneously initiate a bending wave that travels from posterior to anterior direction. The two travelling waves of opposite directions antagonize each other, preventing animals from exhibiting bending or significant forward displacement of the centroid (Fig 2-2A). The *kink* phenotype exhibited upon B ablated animals was reminiscent of, albeit to a decreased degree, to all premotor interneuron ablated animals (Kawano et al., 2011). Our previous and unpublished study showed that during foraging, premotor interneurons exert an inhibitory role on A motor neuron activation (Kawano et al., 2011; Gao et al., in preparation). Together these results indicate
an aberrant activation of A motor neurons in the absence of Bs. Hence, A motor neuron activation may also be inhibited by B motor neurons during forward movement, and such a role is conserved in both L1 and L4 motor circuit.

b. **Forward locomotion exhibits reduced propensity and velocity**

The forward propensity and velocity were considerably reduced in both L1 and L4 animals after the ablation of B motor neurons. At both stages, animals displayed an increase in propensity for pausing, mostly at the expense of forward movement, as well as an overall decrease of forward velocity (Fig. 2-2 B,C). Reduced forward velocity is consistent with an altered bending pattern: a reduced forward movement-driving bending propagation, as well as a failure of suppressing the opposing propagation wave. Hence, B motor neurons sustain forward locomotion in both L1 and L4 animals by both promoting foraging-driving bending wave propagation, and inhibiting the initiation and propagation of reversal-driving bending wave.

c. **Reversal exhibits more substantial difference between L1 and L4 animals**

We observed the most substantial difference between B motor neuron ablated L1 and L4 larvae for all parameters for reversal. B ablated L1s did not exhibit significant changes in the propensity for reversal, whereas B ablated L4 animals exhibited a substantial increased (Fig. 2-2 B). Second, while B-ablated L1s did not alter the initiation frequency to initiate backing, B-ablated L4s exhibited significantly increased backing initiation frequency (Fig. 2-2 D). Third, B-ablated L4, but not L1 animals exhibited higher velocity in reversal.

All results consistently point towards an increased coupling between forward and backward motor circuit as animals develop. While an inhibition from the forward to
backward circuit is consistently from birth, the activity of the backward circuit increases during development.

2.3.1.3. Effect of A-type motor neuron ablation on L1 (DA) and L4 (DA and VA)

In both L1 and L4 animals, the complete ablation of the A motor neurons resulted forward movement with little qualitative abnormalities, but severely impaired reversal. In L4s, A ablation also results in increased forward velocity. This indicated a similar role of A for sustaining reversal during development, but the backward circuit exerts increased inhibition on the forward circuit as animals mature.

a. Ablation of A motor neurons in L1s and L4s drastically reduced reversal

Both A ablated L1s and L4s rarely exhibited reversals (Fig. 2A). Perceived reversal events were when animals paused with temporarily reduced head swings, resuming forward locomotion.

b. A motor neuron ablated L1 and L4 animals increased forward movement

A-ablated L1s exhibited an increased propensity for forward movement, at the expense of both reversal and pausing. However, the mean forward velocity remained unchanged. A ablated L4s, on other hand, while did not change the propensity, exhibited a moderately increased forward velocity (Fig 2-2 C). These results indicate that A motor neurons likely exhibit stronger inhibition on forward locomotion as animals mature, which further support an increased coupling between forward and backward circuits.

2.3.1.4. Effect of D-type motor neuron ablation in L1 and L4

Removing GABAergic D motor neurons lead to reduced velocity for both directional movement in L1s as well as in L4s; but D-ablated L1s exhibited more severely impaired forward movement than in L4s.
a. **Ablating D led to reduced velocity for both directional movement in L1s and L4s**

In both L1s and L4s, the ablation of D motor neurons led to reduced forward movement as well as reversal (Fig. 2-2 B, C). But the impairment was not as severe as A or B ablated animals for reversal and forward movement, respectively. A modest reduction of duration for both forward and backward movement was also observed in D ablated L1s and L4s. The observation that ablation of Ds in L1 affecting both forward movement and reversal was consistent with earlier observation (Chalfie et al, 1985).

b. **D ablated L1s were more severely affected than D ablated L4s**

In D ablated L1s, pausing was increased at a substantial expense of both forward and backward movement propensity. D ablated L4s exhibited only exhibited a modest increase in pausing with slightly decreased forward and backward propensity, suggesting a smaller effect of D motor neurons in maintain locomotion as animals mature (Fig. 2-2 B). While the initiation frequency for forward movement in D ablated L1s was significantly increased, there was not significant change in the initiation frequency for either forward and backward movements in D ablated L4 animals, reinforcing the notion that D motor neurons exert a small impact on forward locomotion as animals mature (Fig. 2D). Consistently, the velocity was also affected more in D ablated L1s than D ablated L4s.

These results suggest that D motor neurons sustain locomotion for both directions, but play a more significant role in L1s. DD motor neurons therefore likely play a more important role in the gait adaption prior to the birth of VDs.
Figure 2-2. A Comparison between the Functional Contribution of Cholinergic and GABAergic Motor Neurons in L1 and L4 Larvae

A. The representative body posture during movement, shown by curvature analyses. The bending angle along the length (Bottom: head, Top: tail) of a moving animal is plotted over time in each panel. **Row 1:** Wild-type animals exhibit primarily continuous forward movement (shown as full head-to-tail wave propagation), and occasional reversal shown as a tail-to-head wave propagation. **Row 2:** Both L1 and L4 animals with all B motor neurons ablated exhibited disrupted forward movement. They exhibited a slower forward gait. Often the bending wave cannot be effectively propagated and diminished in the posterior half of the body. Ablated animals also displayed a kinker phenotype in some cases: during forward locomotion, from the tail of the animal could also initiate a bending wave that traveled from posterior to anterior. **Row 3:** In L1 and L4 animals where all A motor neurons were ablated, they failed to execute long reversal. Attempts to back resulted in a pausing posture with body wall muscles contracted along the body but with no significant displacement. **Row 4:** With DD ablated in L1, animals exhibited deeper bending (deeper color in red or blue). The wave propagation may sometimes discontinue at the anterior body. When all D motor neurons were ablated in L4s, animals exhibited deeper bending near the anterior region, but the wave propagation was only modestly affected.

B. The propensity of directional movements and pausing of indicated genotypes. **In B MN ablated animals:** The tendency to pause was increased with reduced forward movement in both L1 and L4 animals. The B ablation in L1 caused no significant changes in propensity for backward movement, while in B ablated L4 animals the frequency of backward movement was increased. **In A MN ablated animals:** Ablation of A motor neurons in L1s increased the propensity for forward movement, with a reduction in backward movement and pausing. The A ablation in L4s exhibited little change on the temporal distribution of directional movements and pausing. **In D MN ablated animals:** The pausing time increased considerably, with reduced forward and backward movement propensity in L1 ablated animals. In L4 ablated animals, there was a modest increase in pausing, with slight decrease in both forward and backward movement. (N=10 for L1 control, L1 ablated, L4 control and L4 ablated group).

C. The histogram of instantaneous velocity of indicated genotypes. **In B MN ablated animals:** the forward velocity was reduced in both L1 and L4 ablated animals, with L4 animals exhibiting a more severe effect. **In A MN ablated animals:** in L1 animals, the forward velocity was minimally affected by ablated, whereas in L4 animals the forward velocity showed a moderate increase. **In D MN ablated animals:** The instantaneous velocity was greatly reduced in both forward and backward movement, in both L1 and L4 ablated animal.

D. The initiation frequency of forward and backward movement. **In B MN ablated animals:** the backing initiation was not changed in ablated L1s, whereas more backing initiations were observed in L4 ablated animal (P<0.001). **In A MN ablated animals:** no changes were seen in the initiation frequency for forward and backward motions in both L1 and L4 ablated animals. **In D MN ablated animals:** the initiation frequency for forward movement in L1 ablated animals was increased significantly. There was not significant change in the initiation frequency for both forward and backward movements in L4 ablated animals.

E. L1 and adult animal demonstrated similar gait adaptation to viscosity (Credit – E was contributed by Chen S. and Samuel A).
2.3.1.5. Summary and implications

Results from our motor neuron ablation experiments suggested that the role of A, B and D neurons in locomotion output remains largely consistent between L1s and L4s, despite of entirely lacking the L4 ventral muscle innervating subclass motor neurons. Specifically, the cholinergic B and A motor neurons predominantly promote coordinated forward and backward movement, respectively, whereas D motor neurons contribute to the sustainment of movement of both directions. Hence, L1s have to adjust motor circuit properties and/or wiring to maintain a similar output as in L4s.

There are noticeable changes in motor circuit dynamics during development. D motor neurons contribute to the sustainment of movements during development, however, its necessity is reduced as animals mature. Similarly, while ablation of components of the forward circuit promotes reversal and vice versa in both L1s and L4s, the coupling strengthens as animals mature, more noticeably the inhibitory effect of motor neurons that drive reversal on forward-driving circuit. It is possible that a mutual inhibitory mechanism has been imposed between the forward and backward circuits from the excitatory A and B motor neurons in L1s and strengthens over time. Consistently, the D neurons adopt a more supplemental role as the cholinergic motor neuron-driven mutual inhibition becomes gradually established.

2.3.2. Rhythmic B and A motor neuron activity drives forward and backward locomotion, respectively

To specify the activity pattern of cholinergic motor neurons during directional movement
in L1, we examined the activity of each motor neuron classes in moving animals.

2.3.2.1. Experimental methods
The intracellular calcium sensor GCaMP3 or GCaMP6 was used to monitor real-time motor neuron activities and correlated with movement. A ratiometric measurement of co-expressed GCaMP and RFP in transgenic animal’s neurons to offset motion artifact. A GCaMP::RFP fusion protein was used to offset calcium signal readout due to different expression levels amongst individual neurons in the transgenic animals (Kawano, unpublished observations). 5 hours post-hatching L1 animals were used for the recordings, to avoid the critical time period when the ventral class of motor neuron begins to differentiate. Recording was performed on 2% wet agarose pad with a cover slip to reduce its velocity and Z-axis movement.

2.3.2.2. DA motor neuron activation correlates with the contraction of dorsal muscles of the anterior body segment during reversal
Consistent with A motor neurons sustain reversals in L1s, during sustained reversal, DA motor neurons exhibited rhythmic and oscillating calcium level change, following a posterior-to-anterior progression from DA8 and DA9 (most posterior neuron imaged) to DA2 (most anterior neuron imaged) (Fig. 2-3 A). Interestingly, during the initiation of reversal (transition from forward to reversal), the activation order of DAs did not always start at the most posterior segment (DA8 and DA9) (data not shown), but once the reversal is started and persisted, the posterior to anterior propagation was established.

We next examined whether and how DA activity correlates with bending (Fig 2-3 C). In L1s, DAs send anteriorly oriented axons and make NMJs with the dorsal body wall muscle opposing where the anterior DA soma resides (Daniel Witvliet, unpublished
results). Should DA drive dorsal muscle contraction through those NMJs, DA$_N$ activity increase should coincide with increased curvature at position of the DA$_{N-1}$ soma (Fig 2-3 D). We performed a correlation analysis between the DA$_N$ activity and the curvature at DA$_{N-1}$, DA$_N$ and DA$_{N+1}$. Not only did the DA$_N$‘s activity increase exhibited the least temporal lag with curvature increase at DA$_{N-1}$ soma, it also exhibited the highest correlation value with the curvature increase at DA$_{N-1}$ soma (Fig 2-3 E).

2.3.2.3. **DB motor neuron activation correlates with contraction of dorsal muscles of the posterior body segment during forward movement**

Similarly, we examined the activity pattern of the B-class motor neurons and their correlation with body bending by real-time calcium imaging. During forward movement, we observed sequential, rhythmic and cyclic activation of DB motor neurons in an anterior to posterior order (Fig 2-4 A, E). Mirroring to the anatomic tiling of DA motor neurons, the NMJs of DB$_N$ neurons are clustered dorsally, near the posterior body segment where the soma of DB$_{N+1}$ resides (Daniel Witvliet, unpublished results). Also mirroring the activity profile of L1 DAs, the calcium rise in DB$_N$ exhibited the shortest temporal lag and highest correlation with an increase of curvature at DB$_{N+1}$ position (Fig. 2-4 C, D). Hence L1 DB activation correlated with contraction of the dorsal muscles posterior to its soma, coinciding with their axons.

In summary, L1 DA and DB motor neurons participate in the two sub-circuits that are dictated for forward and backward locomotion, respectively. Their activities likely directly innervate dorsal body wall muscle contraction during the directional movement.
Figure 2-3

A

B

C

D

E
Figure 2-3. DA motor neuron activation correlates with the contraction of dorsal muscles at the anterior body segment during reversal

A. A set of representative calcium transient traces in DA motor neurons during semi-constrained locomotion. Top panel indicated the velocity of the animal, with the value above and below the zero line indicate forward and backward movement, respectively. During a period of sustained backing, a posterior-to-anterior progression of DA neuron activities was observed (From DA8/9 to DA2, in shaded area).

B. An illustration of DA neuron position. From anterior to posterior are DA2 to DA8/9.

C. Qualitative comparison between calcium transient of DA4 soma (Left y-axis, black line) with the body-bending angle (for calculation method, see Figure 2-11) of DA3, DA4 and DA5 soma region (Right y-axis: blue lines). Shaded boxes indicate the backing periods. Angle > 180 degree represents dorsal contraction. The calcium level in DA4 soma represented by GFP/RFP ratio more tightly correlated with bending of DA3 soma region.

D. A schematic illustration of DAn activity correlating with the anterior dorsal muscle parted by where the DAn-1 soma resides. E. The cross-correlation of DA4 activity and bending angle of the regions marked by DA3, DA4 and DA5. The value is highest at DA3 (0.8048) at the lag (0s). DA4 activity exhibited the highest correlation with dorsal contraction at its anterior region.
Figure 2-4. DB motor neuron activation correlates with contraction of dorsal muscles of the posterior body segment during forward movement

A. Representative calcium transient traces in DB motor neurons during semi-constrained forward locomotion. An anterior to posterior progression of DB neuron activities was observed (From DB3 to DB7).

B. Demonstration of DB neuron position during imaging. Anterior to posterior: DB3 to DB7.

C. Qualitative comparison between calcium transient of DB5 soma (Left y-axis, black line) with the body bending angle of the DB4, DB5 and DB6 soma region (Right y-axis: blue lines) during a period of forward movement. Angle > 180 degree represents dorsal contraction. The calcium level in DB5 soma represented by GFP/RFP ratio tightly correlates with the body bending of DB6 soma region.

D. Top: A schematics representing that the DBn activation correlates with the contraction of the posterior dorsal muscle near the DBn+1 soma region. Bottom: The cross-correlation of DB5 activity and bending angle of DB4, DB5 and DB6 soma region. The correlation value is highest at DB6 (0.5061) at the lag=1s. The activity of DA4 exhibited the highest correlation with dorsal bending at DA3 soma region. E. During backward locomotion (Shaded box), DB motor neurons did not show noticeable increase of calcium transients.
2.3.3. **DD motor neurons exhibit rhythmic oscillating activities that correlate with relaxation of ventral muscles during both forward and backward movement**

We performed real-time calcium imaging of the L1 DD motor neurons. Differing from the case for DA and DB, the morphology of DD neuron allowed me to image the axonal regions directly (Fig. 2-5 B). Calcium increase in L1 DD ventral processes directly correlated with local, ventral relaxation, and DDs exhibited sequential, similar, anterior to posterior activation during forward movement, and posterior to anterior activation during reversal. Importantly, DD activity is dependent on DB and DA activation during forward movement and reversal, respectively.

2.3.3.1. **Rhythmic DD activation patterns correlate with ventral muscle relaxation during forward and backward movement**

DD axons exhibited a rhythmic activity pattern that is tightly coupled with undulation. They exhibited anterior to posterior progression during forward movement, whereas the oscillating activity of the DD$_n$ axon exhibited a phase offset with that of DD$_{n-1}$ axon. During reversal, the activity pattern was reversed, and propagating in a posterior-to-anterior order (Fig.2-5 C). When animals transited between directional movements, DD activation transgressed effectively to reverse the bending wave – the mode of transition, often involving either adjusting the phasic relationships between multiple DDs (Fig.2-5 E). These results support a cross-inhibitor role for the L1 D-type motor neurons on ventral muscles.

A key question is whether the rhythmic L1 DD activity mediates an excitatory or inhibitory effect on the ventral muscles. Calcium imaging performed at DD axons allowed us to directly correlate the local calcium transient level with bending at the
ventral body segment. Our cross-correlation analysis between the change of calcium level and angle of the body segment encompassed by the axonal region showed that the activity of each DD axon positively correlated with the angle, an index for elongation in the innervated body segments. Hence DD motor neurons likely mediate an inhibitory effect on ventral body wall muscles, causing relaxation (Fig. 2-5 D).

2.3.3.2. DD activity is dependent on inputs from DB and DA during forward and backward movement, respectively

In the adult connectome, D motor neurons receive direct inputs from the A- and B-type motor neurons, indicating their activity dependence on cholinergic motor neuron inputs (White et al., 1976). To determine whether L1 DD’s activity is dependent on inputs from DA and DB motor neurons, I utilized an approach where I combined motor neuron calcium imaging and optogenetic ablation. Briefly, the DA and DB motor neurons were ablated using miniSOG 40 minutes after hatching, and the DD motor neuron axon activity was recorded 2 to 3 hours after the ablation.

When DA neurons were ablated, when animals moved forward, DD axons exhibited normal activity level, propagating sequentially in an anterior to posterior order (Fig. 2-6 A). During ‘reversals’, where animals attempted to go backwards and a long pause was observed, DD axon activity was diminished when compared to the activity during forward movement during same period of recording (n=6, p=0.03 A pair-wise T test) (Fig. 2-6 C).

Conversely, when DB motor neurons were ablated, although animals could still generate sluggish forward movement, the calcium transient in DD axons was greatly
reduced (n=6, p=0.0025 by pair-wise t test) (Fig. 2-6 B, C). During reversals, DD axons exhibited high rhythmic DD activity that follows a posterior to anterior propagation order as in non-ablated control animals.

These results suggest that in L1s, DD activities depend on DB’s input during forward locomotion and DA during reversal. A reasonable postulation is that inputs from both DB and DA motor neurons dictate D’s oscillating activity during forward and backward movement, respectively, and reset their phasic relationships when animals transit between directional movements. It remains to be determined whether DA and DB function synergistically to coordinate such transitions.
Figure 2-5. D motor neurons exhibit rhythmic oscillating activities that correlate with ventral muscle relaxation during both forward and backward movement

A. A schematic diagram of the DA, DB and DD module.

B. An illustration of the region of interests (ROI) selection for DD neuron imaging: the DD axonal region can be traced using the program. They reside at the anterior side of DD soma.

C. The activity pattern of DD neurons during both directional movements in L1 animals. Top panel: velocity of the animal where the velocity above and below zero represented forward and backward movement, respectively. Middle panel: During forward movement, the axon activity demonstrated a clear anterior to posterior sequence from DD3 to DD5 axons. During backing period, on the other hand, the sequential firing pattern was reversed to be posterior to anterior order (shaded box). Bottom panel: The body wave propagation from anterior to posterior direction was demonstrated by the angle of local body bending (angle at DD axon) as a function of time. The angle above 180 represents the ventral relaxation while the angle smaller than 180 represents ventral contraction. As shown, during forward movement, for body regions that cell soma and axon resides, respectively, the body bending wave follow and anterior to posterior direction. For the backward period, the body wave showed a posterior to anterior direction.

D. Cross-correlation analysis was used to determine the temporal lag of local body bending angle to axon activity during a period of forward locomotion. The lags are found to be positive for all body bending to axon activity.

E. Cross correlation of DD4 vs. DD3 axon activity, DD5 vs. DD4 axon activity and DD6 vs. DD5 axon activity during forward and backward locomotion. A positive lag was seen between more posterior DD axon to more anterior DD axon during forward movement, and a negative lag was seen during backward movement. This confirmed that DD axon follows anterior to posterior activation sequence during forward and a reversed pattern during backward locomotion.
Figure 2-6

A

B

C

Control DD activity

DD activity with DB ablated

DD activity with DA ablated

Average Activity

Forward

Backward

Forward

Backward

p=0.0025

p=0.03
Figure 2-6. DD activity is dependent on inputs from DB and DA during forward and backward movement, respectively

A. When all DB motor neurons are ablated, the DD motor neuron’s axon activity was greatly reduced during forward locomotion. During frequent backing, DDs maintained relatively normal activity.

B. When DA neurons were ablated, DDs activity was normal during forward locomotion, and diminished during the “backing attempts” (no body displacement).

C. A pair-wise comparison of average activity DD4 MN during forward and backward movement in DB MN ablated and DA MN ablated animals (pair-wise t test, n=6). DD4 activity was significantly reduced during forward movement upon DB ablation compared to backing (p=0.0025). DD4 activity was significantly decreased during forward locomotion compared to backward locomotion upon DA ablation (p=0.03).
2.3.4. An intrinsic difference between ventral and dorsal muscle does not account for undulation

Because DD motor neurons impose inhibitory inputs on ventral muscles, a key question arises on the source of excitatory inputs that mediate ventral muscle contraction. We propose three hypotheses on how L1 generates alternating ventral/dorsal bending in the absence of direct excitatory inputs to ventral muscles: First, ventral body wall muscles are intrinsically more active than dorsal muscles; by default, ventral muscles are constitutively contracted, hence inhibitory inputs from DDs were sufficient to generate undulation. Second, other unidentified neurons provide excitatory inputs to ventral muscles. Third, a crosstalk between dorsal and ventral muscles, through synaptic or asynaptic communications, orchestrates their reciprocal inhibition. These mechanisms are not mutually exclusive, allowing multiple layers of regulation on the L1 motor circuit.

2.3.4.1. Summary of experimental methods

Two sets of experiments were carried out to examine whether ventral muscles exhibit higher intrinsic activity than dorsal muscles; such a property would predict that in the absence of all motor neurons, L1s are constitutively contradicted ventrally.

The first approach is a swimming assay. Comparing to on plate assay (where animals crawl), the innate muscle activity is more readily observed in liquid of low viscosity (where animals swim). We quantified parameters that result from asymmetric ventral and dorsal muscle activity – ventral or dorsal coils. A coiled or omega shaped body posture is generated when an L1’s head or tail comes into contact with its body on either the dorsal or ventral side. We define the duration of the body contact as the dorsal or ventral bending period, respectively. We observed that L1s use omega turns to change
swimming direction; such turns often result from the head transiently contacting the ventral body before finishing the turn (Movie M1). We separated score the turning events from the ventral or dorsal bending.

The second approach is to directly access the body wall muscle activity by calcium imaging in the absence of DA, DB and/or DD motor neurons. Transgenic lines that express miniSOG in respective motor neurons and GCaMP::RFP in body wall muscles were used. Motor neuron ablation was performed within 1 hour post hatching and calcium imaging was performed within 6 hours after hatching. Dorsal and ventral muscle activity was separated and examined for their propagation and level.

2.3.4.2. Ablation of all motor neurons results in unbiased ventral and dorsal coils in liquid

We ablated all DA, DB and DD motor neurons in L1 animals by co-expression miniSOG under their respective promotors synchronously, and examine the muscle activity by observing the swimming behavior. As shown in Fig. 2-7, ablation of all L1 motor neurons, animals exhibited an increased the propensity and duration for both ventral and dorsal bending. These animals retained head swing, yet the body often acquired a stringent coil onto either ventral or dorsal sides. The tendency for ventral or dorsal coil did not exhibit a particular bias, neither in initiation frequency or length of the exacerbated bending (Movie M2, Fig 2-7 A).

unc-13(s69) is a severe loss-of-function mutant with disrupted neurotransmitter release. If there is a difference between the intrinsic activity of ventral and dorsal body wall muscles, unc-13 mutants should not exhibit no bias on ventral or dorsal coils. In liquid, unc-13(s69) animal lacked head swing, and exhibited drastically increased
duration of prolonged ventral or dorsal bending, resulting a smaller number of ventral or
dorsal bending event during the recording period than motor neuron ablated L1s (Movie
M3, Fig 2-7 A). Regardless of the counts, neither *unc-13* nor motor neuron ablated L1s
exhibited biased ventral coil counts (N=10 for each condition).

2.3.4.3. Ablation of all motor neurons equally reduced calcium transient in both
dorsal and ventral muscles

We further assessed ventral and dorsal muscle activity in all motor neuron ablated and
*unc-13*(S69) mutant L1s by real-time calcium imaging (Lu Y., unpublished results).
Consistent with an alternating dorsal and ventral muscle activation and relaxation, in
control (no ablated or wild-type) L1 animals, the dorsal and ventral body muscle
posterior to the head exhibited alternating activation and inactivation (Fig 2-7 B). The
increase of calcium signal on either side correlated with its contraction. The calcium
transient also propagated in the same direction of dorsal or ventral ben propagation. The
mean of activity level on the ventral and dorsal, as expected from unbiased bending,
exhibited an approximate ratio of 1 (Fig 2-7 B).

Under the same recording conditions, in both *unc-13* and motor neuron ablated
animals, mean muscle calcium signals were reduced, while the residual calcium signals
exhibited ‘spasm’, instead of propagation pattern (Fig 2-7 C). The residual calcium
activity was also insufficient to driven physical displacement of the animal, which is
consistent with the observed prolonged ventral and dorsal coils by the swimming assay.
An important finding was that in the absence of motor neurons (ablated), or, synaptic
transmission (*unc-13*), both ventral and dorsal muscle activity was equally affected (Fig.
2-7 C). The ratio of remaining mean calcium activity of ventral and dorsal muscles, as in
control animals, was close to 1.

Therefore, both behavioral analyses and muscle calcium imaging experiments support the notion that three types of L1 motor neurons plays an important role, directly or indirectly, in mediating both ventral/dorsal wall muscle activity, and ventral/dorsal bending, and there was no obvious difference in the ventral and dorsal muscle activity in the absence of motor neuron inputs.
Figure 2-7. Ablation of all motor neurons results in unbiased ventral and dorsal muscle activity

The in-liquid assay measured the prolonged ventral or dorsal coils to quantify and compare the levels of ventral and dorsal muscle activity. The duration (average and cumulative in all animals tested) and counts were used to represent the time and frequency that animals spent in dorsal or ventral coiled posture in liquid. We observed that L1s use omega turns to change swimming direction; such turns often result from the head transiently contacting the ventral body before finishing the turn. We separated score the turning events from the ventral or dorsal bending. *Unc-13*(s69) animals did not execute any turning during recording, therefore there was no need to separate it.

A. Upon ablation of all L1 motor neurons, animals exhibited an increased the propensity and duration for both ventral and dorsal bending. The tendency for ventral or dorsal coil did not exhibit a particular bias, neither in initiation frequency or length of the exacerbated bending. The *unc-13*(s69) mutant did not exhibit bias on ventral or dorsal coils. (N=10 for each condition).

B. Ventral and dorsal muscle activity in all motor neuron ablated and *unc-13*(S69) mutant L1s by real-time calcium imaging. *Left panel:* In control L1 animals, the dorsal and ventral body muscle posterior to the head exhibited alternating activation and inactivation, corresponding to alternating dorsal and ventral muscle activation and relaxation shown in curvature plot. The calcium transient also propagated in the same direction of dorsal or ventral bending. *Middle panel:* Upon all DA, DB and DD motor neuron ablation, mean muscle calcium signals were reduced in the animal while the residual calcium signals on both ventral and dorsal side. The muscle exhibited spasm-like calcium transient, instead of propagation pattern. *Right panel:* The mean of activity level on the ventral and dorsal, as expected from unbiased bending, exhibited an approximate ratio of 1.

C. *Left and Middle panel:* *Unc-13*(s69) mutant demonstrated reduced level of and disrupted propagation of calcium transient on both dorsal and ventral side. *Right panel: Top:* The average dorsal/ventral activity ratio was not changed significantly in *unc-139*(s69) mutant compared to contl. *Bottom:* The ratio of wild-type/*unc-13* ventral and dorsal muscle activity was similar.
2.3.5. Both ventral and dorsal muscle innervation requires DA and DB motor neuron inputs for undulation

To determine which excitatory motor neurons were required for ventral muscle contraction, we separately ablated the inhibitory DD motor neurons, and the cholinergic (DB and DA) motor neurons, and accessed their effect by both swimming assays and muscle calcium imaging (when possible).

2.3.5.1. DD motor neurons are not required for ventral muscle contraction

Upon DD ablation, L1 animals remained active in locomotion, albeit with reduced velocity (Fig 2-8 B). Unlike all motor neuron ablated L1s, however, they exhibited a strong bias for prolonged dorsal bending (Movie M4). This observation further supports the DD axon calcium imaging data, where its activity increase correlated with an increased body angle (ventral relaxation), and indicates that the DD motor neurons activity was strictly coupled with an inhibition of ventral muscle contraction. We did not perform muscle calcium imaging due to failures to obtain transgenic strains co-expressing DD neuron specific miniSOG and muscle specific GCaMPs. However, the swimming assays indicate that ventral muscle contraction does not require, and is inhibited by DD motor neurons.

2.3.5.2. DA and DB motor neurons are required for ventral muscle contraction

In contrast, the ablation of DA and DB motor neurons resulted in L1 animals with remarkable similarity to all motor neuron ablated animals, in both swimming assays and muscle calcium imaging analyses.

In brief, DA and DB ablated L1s exhibited an increased number as well as duration of prolonged ventral and dorsal bending when compared to non-ablated L1s (Fig
2-8 A; Movie M5). Critically, there was no bias between ventral and dorsal coiling. Similarly, while the mean muscle calcium transient level was reduced in ablated L1s, the reduction did not exhibit a ventral/dorsal bias (Fig. 2-8 C).

Therefore, DA and DB, the only known cholinergic motor neurons in L1 larvae are required, albeit lacking direct NMJ inputs to ventral muscle activation. Hence in parallel to the remodeling of GABAergic DD motor neurons, the cholinergic motor neurons may also undergo property or wiring changes as animals develop.

2.3.5.3. Summary of results

First, DA, DB and DD motor neurons accounts for the muscle calcium activity that drives movement. In their absence, both dorsal and ventral muscles exhibited residual calcium activity, but it was insufficient to drive undulation. Second, in both the presence and absence of all L1 motor neurons, dorsal and ventral muscle activity level is similar. Ventral body wall muscles do not exhibit constitutively higher intrinsic activity than dorsal muscles. Third, GABAergic DD motor neurons are inhibitory, driving ventral muscle excitation. Cholinergic DA and DB motor neurons are required, directly or indirectly, for contraction of both dorsal and ventral muscles. Based on our current partial L1 EM reconstruction, it is highly unlikely that DB or DA motor neuron forms direct NMJs onto ventral muscles (White et al., 1978; Zhen lab, unpublished). Hence, cholinergic motor neurons are more likely to provide indirect input onto ventral body wall muscles.
Figure 2-8

A

Average duration

Total duration

Total counts

Control vs. DA+DB Ablated

B

Average duration

Total duration

Total counts

Control vs. DA+DB Ablated

C

Control vs. DA+DB Ablated

Dorsal GaMeP intensity

Ventral GaMeP intensity

Curvature

Time/s
Figure 2-8. Both ventral and dorsal muscle contraction requires DA and DB motor neuron inputs

A. Upon ablation of all DA and DB motor neurons in L1, animals exhibited an increased the propensity and duration for both ventral and dorsal coils. The tendency for ventral or dorsal coil did not exhibit a particular bias, neither in initiation frequency or duration of the exacerbated bending.

B. Ablation of DD motor neurons in L1 results a strong bias for prolonged dorsal bending.

C. Ventral and dorsal muscle activity in DA and DB motor neuron ablated animals by real-time calcium imaging. *Left panel:* In control L1 animals, the dorsal and ventral body muscle posterior to the head exhibited alternating activation and inactivation, corresponding to alternating dorsal and ventral muscle activation and relaxation shown in curvature plot. *Middle panel:* Upon all DA and DB motor neuron ablation, mean muscle calcium signals on both ventral and dorsal were reduced. *Right panel:* The ratio of doral/ventral activity level between control and DA+DB ablated animals are not significantly changed.
2.4. Discussion

2.4.1. Summary of results and implications

In this study, we investigate the neural network activity responsible for directional movement in the C. elegans newborn larvae (L1s) prior to the first round of post-embryonic neurogenesis. These animals have a ‘juvenile’ circuit that consists of 202 neurons including 22 motor neurons. Little is known about the synaptic connectivity and the motor circuit activity pattern that underlies undulatory movement exhibited by L1 animals.

Through a combination of neurophysiology, optogenetics and behavioral studies, we found that the cholinergic B- and A-type motor neurons exhibits rhythmic activity increase that correlates with muscle contraction and propagation during forward and backward movement, and an ablation of the DB or DA motor neurons severely impaired the forward and backward movement, respectively. Both experiments denote their prominent contribution to directional movement. We further determined their activities are required for both ventral and dorsal muscle contraction.

The rhythmic DD motor neuron activity on other hand, drive ventral muscle relaxation during both forward and backward movements. Hence GABAergic transmission remains of inhibitory property throughout development. During forward and backward movement, DD activity is strictly dependent on inputs from the DB and DA cholinergic motor neurons, respectively.

Our studies did not resolve a direct source for ventral body wall muscle excitation and dorsal body wall muscle relaxation. But we ruled out the possibility that an intrinsic
difference in the default ventral versus dorsal muscle activity compensates for asymmetric excitatory versus inhibitory inputs to ventral and dorsal muscles. A consistent, but unexpected implication throughout the study is that the ventral body wall muscle contraction also requires DA and DB motor neuron activation, despite of a lacking of direct NMJ outputs to the ventral muscles.

We propose that the L1 ventral muscle activation relies on unidentified intermediate neurons (neuron X) that are activated by cholinergic DA and DB motor neurons, and L1 dorsal muscle relaxation is the default state upon DA and DB inactivation (Fig 2-9). An accurate and complete motor circuit wiring diagram of the L1 animals is necessary and will provide critical insights to test these hypothesis.
Figure 2-9. Proposed mechanism underlying ventral body bending in L1 C. elegans

A diagram showing postulated mechanism of L1 undulation. Cholinergic motor neurons DA and DB mediate direct muscle contraction of dorsal muscle. Ventral muscle activation relies on unidentified intermediate neurons (neuron X) that are activated by cholinergic DA and DB motor neurons. The L1 dorsal muscle relaxation is the default state upon DA and DB inactivation, and the ventral muscle relaxation requires DD mediated inhibition.
2.4.2. A proposed operation model of an L1 motor circuit

What are the potential mechanisms that allow cholinergic motor neurons to activate ventral muscles in the absence of direct NMJs? In the absence of data, we could only retreat to speculations. We speculate the presence of an unidentified and uncharacterized, indirect circuit connection or synaptic transmission to allow DA and DB motor neurons to regulate ventral muscle activity. This ‘indirect’ communication may contain one or more layers of intermediate neurons between motor neurons to ventral muscles: upon activation by DBs and DAs during directional undulation, they an increased activity that in turn innervates ventral muscles (Fig. 2-9). The DD motor neurons, receiving dyadic input from DB and DA, in turn mediate inhibitory signal to the segmental ventral muscles opposing the contracting dorsal muscles to facilitate ventral and dorsal bending (Fig. 2-9). Gap junctions between neurons and muscles, a reported prominent feature for the developing circuits (Fulton, 1995; Roerig and Feller, 2000), may provide an additional resource for indirect innervation of ventral muscles through these motor neurons. Currently, we are carrying out a complete reconstruction of the L1 sensory motor circuit to allow the screening for candidate neurons or muscle connections.

2.4.3. DA, DB and DD motor neurons provides the main, but not sole inputs to muscle activity

Another unexpected finding was that upon the cellular removal of all known L1 motor neurons, we still observed calcium transients in both ventral and dorsal body wall muscles, albeit with much reduced level. The kinetics of residual activity at dorsal and ventral muscles differs from that of the DA, DB and DD-driven calcium profiles: they
propagate bi-directionally, almost simultaneously, exhibit ventral dorsal alternation, and do now generate sufficient or coordinated muscle force to mediate directional movement.

This activity, if is of neuronal origin, must result from circuit activities independent of the known L1 motor neurons. We could not exclude the possibility of a spontaneous muscle activity, but their ventral and dorsal alternation pattern hints neuronal modulation. If they are of neuronal origin, the fast propagation of calcium signals along the entire body suggests that these unidentified neurons likely have axon expanding the entire length of the ventral and dorsal nerve cords. Interestingly, upon the genetic ablation of chemical synaptic transmission of the entire nervous system (unc-13(s69)), we also observed residual calcium signal in both dorsal and ventral body wall muscles, but with slightly different features. The residual activity is also spasm-like, quickly spread anteriorly and posteriorly simultaneously. Unlike the case for motor neuron ablated animals, however, the dorsal and ventral muscle activities did not exhibit alternating phasic relationships (Fig. 2-7 C, Y. Lu, unpublished results).

Such a difference suggests that additional mechanisms are in place to generate reciprocal inhibition between dorsal and ventral body wall muscle activation, and such a mechanism is independent of the lateral-inhibition module proposed for the cholinergic-GABAergic motor neuron circuit. This mechanism is disrupted in unc-13(s69) mutants, indicating a requirement for neuronal inputs and chemical synaptic communication or secretion. Hence, multiple layers of mechanisms are involved in the temporal and spatial coordination of body muscle activity of the L1 motor circuit. It will be of interest to examine whether multi-layer regulation is replaced upon the maturation of the lateral-inhibitory motifs for both ventral and dorsal muscles in adults, or, they are still present in
the mature motor circuit.

2.4.4. A developmental transition of motor circuit function in C. elegans

2.4.4.1. An activity pattern change of GABAergic motor neurons during development

DD motor neurons exhibit the most profound anatomical changes between the L1 and adult motor circuit, with a complete reversal of their axon-dendrite polarity (Hallam and Jin, 1998; Walthall et al., 1993; White et al., 1978). In this study, I identified a previously unreported activity pattern change.

In L1s, DD axons exhibit a similar activity pattern during forward and backward movement. In older larvae (L3 and L4), DDs have completed anatomic remodeling and VD motor neurons have been born and incorporated in the motor circuit. I consistently observed a drastic increase in calcium level was observed for DD (and the post-embryonic derived VD) motor neurons during backward movement, whereas the change was fairly modest during forward movement (Fig. 2-10 A, B).

These results suggest that the maturation of VD motor neurons may trigger a change in the functional contribution of the GABAergic motor neurons in directional movement. Specifically, they become less essential for coordinated muscle activity during forward locomotion when animals develop. This finding seems to assert, in contrary to previous hypothesis, that reciprocal and lateral inhibition is not central and necessary for some mode of sinusoidal locomotion. In this regard, I shall raise caution to a technical caveat for drawing conclusions based on a direct comparison of the L1 and L3 motor neuron imaging results: in L1 animals, the imaging was performed at DD axons,
likely at synapse-clustered regions, whereas in L3 animals, I was only able to reliably image DD soma. Hence, a possibility remains where in L3 stage animals, the tonic calcium level in D motor neuron soma correlate with the direction of locomotion, while the axonal activity are rhythmic and differentially regulated during both directional movement (Fig. 2-10 A).
Figure 2-10

A

L3

B

L4
The activity pattern of the GABAergic D motor neurons in later development.

The activity patterns of DD and VD neurons in L3 and L4 animals during locomotion. Top panel indicated the relative velocity of the animal, the velocity above zero indicate forward and backward movement, respectively. VD3, DD2 and VD4, from anterior to posterior, were imaged in L3 and L4 stage animals.

A. DD/VD activity in L3 animals. In L3, The activity of the DD2, VD3 and VD4 neurons all showed an overall increase during backing period, while when maintained or switch to forward movement, the activity of the D MNs was low.

B. Similarly in L4 animals, the activity of DD3, VD3 and VD4 increased during backward movement and decreased in forward movement.
2.4.4.2. Interaction between the forward and backward circuits during development

Our motor neuron ablation results revealed a previously unknown interaction between the forward and backward motor neurons as the motor network matures post-embryonically. In L1s, the DB and DA motor neurons function relatively independently to promote forward and backward locomotion, respectively. However, in later stages, the forward and backward circuit exhibited increased mutual inhibition, or more coupling.

By contrast, inhibitory motor neurons retrieve to a more supplementary role for coordinating undulation as animals develop. As D motor neurons receive synaptic inputs from the A and B class cholinergic motor neurons, a distinction between the D motor neuron activity in forward and backward movement during motor circuit development may also reflect a functional changes of the excitatory of cholinergic A and B MNs. One noted feature from the ablation studies was a potential increase of the activity of A motor neurons in older adults, and if in old adults D motor neuron activation is also dependent on A motor neuron inputs during reversals, then this would be consistent with the observed higher D activity during backing than during forward movement.

It is conceivable that during development, this new layer of mutual inhibition become the foundation for animals to develop a more sophisticated control mechanism on their gait and sensory-motor response, such as promoting a bias towards forward locomotion (Kawano et al., 2011; Zheng et al., 1999). This change in circuit activity during the active modification of connectome within the motor network, suggesting a systematic and adaptive maturation during development, the cellular mechanism of which are yet to be characterized.
2.4.5. **Comparison with other systems**

During the development, animals need to adapt to the constant rearrangement of the cellular components of a developing nervous system, while maintaining desirable motor output suitable for their environment. Albeit the difference in anatomic organization between limbed and non-limbed animals, there should be common principles in strategies that animals utilize to coordinate circuit development and behavior output.

2.4.5.1. **Reciprocal inhibition of motor circuits that generate mutually exclusive behaviors**

In this study, we obtained evidence for a lateral inhibition and reciprocal inhibition mechanism that underlies the directional undulation pattern in developing *C. elegans*. The dorsal muscles are activated by the rhythmic activity of cholinergic DB and DA motor neurons, in an anterior-to-posterior and posterior-to-anterior order during forwarding and reversal, respectively. Simultaneously, DA and DB drive the rhythmic DD motor neuron activity to relax dorsal ventral muscles while the dorsal muscles contract in the same body segment. This reciprocal inhibition of dorsal and ventral muscle groups through the interplay between cholinergic motor neurons and GABAergic motor neurons is comparable, in principle, to the contra-lateral inhibition mechanism in the vertebrate left-right coordination during walking.

In newborn rodents, the rhythm-generating circuit provides inputs onto some inhibitory commissural interneurons (CINs) that directly project onto the contralateral motor neurons, and some excitatory CINs activate neurons on the contralateral side that inhibit the same motor neuron groups (Quinlan and Kiehn, 2007). Dual mechanisms by CINs cause inhibition of the contralateral motor neurons to secure left-right alternation.
In non-limbed tadpole and lamprey, a cross-inhibitory network consisting of two inhibitory CINs that impose mutual inhibition facilitates the left-right alternation in swimming (Kiehn, 2011). These systems lack the excitatory CINs and therefore exhibit a single layer reciprocal inactivation (Kozlov et al., 2009).

I propose that *C. elegans* DD motor neurons serve as a partial functional analogue to CINs. In L1s, the inhibition of the ventral muscle is coupled with simultaneous excitation of dorsal muscle in the same body segment because DD motor neurons receive direct input from the excitatory motor neurons that innervates dorsal muscles. Given the relatively minor changes in movement pattern in DD ablated L1s, DD feedback is not necessary for oscillating DA and DB activation, but modulating DA and DB output.

### 2.4.5.2. The role of GABAergic signaling in the developing motor circuits

A profound switch from excitatory to inhibitory nature of GABAergic synaptic transmission was considered a conserved feature in animal species. In the immature mammalian CNS, GABA exerts excitatory postsynaptic response prior to the maturation of glutamatergic synapses, a process governed by the reversal of chloride potentials (Ben-Ari, 2002; Ben-Ari et al., 1989; Rivera et al., 1999). The depolarizing effect of GABA was regarded as an essential drive for circuit development (Cancedda et al., 2007).

It was reported recently that the *C. elegans* DD GABAergic synapses may also switch from being excitatory to inhibitory at mid-L1. This was based on their response to muscimol, a GABA receptor agonist, where its application in before mid-L1 led to ventral muscle contraction (Han et al., 2015). However, they postulated an inhibitory
nature of endogenous GABA release upon ChR2 stimulation, contributing the
discrepancy in an animal’s responses to muscimol bathing vs. ChR2-stimulated GABA
release to differences between activating all versus postsynaptic GABA receptors.

My calcium imaging studies point towards GABAergic synapses by DD motor
neurons of inhibitory nature from birth to adulthood. In line with their finding, I have
shown that the activity of DD motor neurons correlates with relaxation of ventral body
wall muscle. However, I identified a different form of activity during post-embryonic
maturation, where they transit from exhibiting equal activity level between forward and
backward locomotion, and contribution to both forward and backward movement in L1s,
to a higher activity and more critical role in reversal in later adults. These results suggest
that the remodeling of GABAergic system may undergo different, and perhaps more
complex process that previously assumed.

In summary, we have begun to characterize how during development, *C. elegans*
may generate a similar locomotion pattern with a different circuit composition. Through
this and ongoing study by other members of the lab, we hope to gain insights on general
principles that govern circuit and behavioral maturation across species.
3. Material and Methods

3.1. Construct and strains

All *C. elegans* strains used were cultured and maintained at 22°C using standard methods on Nematode Growth Medium plates (NGM) seeded with OP50 *E. coli* as food source (Brenner, 1974). Unless otherwise stated, Bristol N2 strain was treated as standard wild-type strain for behavioral analysis. Null mutant *unc-7(e5), unc-13(s69)* obtained from *Caenorhabditis* Genetics Center (CGC) (USA) were used throughout the study.

Transgenic animals carrying extra-chromosomal arrays (*hpEx*) were created by co-injecting the DNA plasmid of interest and a co-injection marker at 5-30 ng/µL. All strains used for calcium imaging and behavior analysis were prepared by co-injecting target plasmid DNA with a *lin-15* rescuing plasmid into *lin-15(n765)* animals. Subsequently, the extrachromosomal arrays were integrated into the genome using UV irradiation method to create stable transgenic lines (*hpIs*) (Mello et al., 1991). All integrated lines were backcrossed at least 4 times against wild-type N2 strain.

Table 1. A list of constructs and transgenes generated for Chapter 2

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Transgene</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Imaging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJH2999</td>
<td><em>Punc-25-GCaMP3::wCherry</em></td>
<td><em>hpIs536</em></td>
<td>ZM7656</td>
</tr>
<tr>
<td>pJH3137</td>
<td><em>Punc-4-GCaMP6::wCherry</em></td>
<td><em>hpIs459</em></td>
<td>ZM8428</td>
</tr>
<tr>
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<td><em>Pacr-2(s)-GCaMP6::wCherry</em></td>
<td><em>hpIs595</em></td>
<td>ZM9109</td>
</tr>
<tr>
<td>Neuro Ablation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJH2842</td>
<td><em>Pacr-5-tomm-20::miniSOG-urSL-wCherry</em></td>
<td><em>hpIs372</em></td>
<td>ZM7798</td>
</tr>
<tr>
<td>pJH2843</td>
<td><em>Punc-4-tomm-20::miniSOG-urSL-wCherry</em></td>
<td><em>hpIs371</em></td>
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</tr>
<tr>
<td>pJH2844</td>
<td><em>Punc-25-tomm-20::miniSOG-urSL-wCherry</em></td>
<td><em>hpIs377</em></td>
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<tr>
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<td><em>Punc-4-tomm-20::miniSOG-urSL-wBFP</em></td>
<td><em>hpIs559</em></td>
<td>ZM9126</td>
</tr>
<tr>
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<tr>
<td>pJH3626</td>
<td><em>Pacr-2(s)-tomm-20::miniSOG-urSL-wCherry</em></td>
<td><em>hpIs583</em></td>
<td>ZM9062</td>
</tr>
<tr>
<td>pJH3627</td>
<td><em>Pacr-2(s)-tomm-20::miniSOG-urSL-wBFP</em></td>
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</tr>
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</table>
Strains used in Chapter 2

**Genetic mutants**

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<tr>
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<th>Mutation</th>
</tr>
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<tbody>
<tr>
<td>ZM8661</td>
<td>unc-13 (s69)</td>
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<tr>
<td>CB156</td>
<td>unc-25 (e156)</td>
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**Genetic ablation:**

<table>
<thead>
<tr>
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<th>Insertion</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>hpIs371</td>
</tr>
<tr>
<td>ZM7697</td>
<td>hpIs377</td>
</tr>
<tr>
<td>ZM9075</td>
<td>hpIs540</td>
</tr>
<tr>
<td>ZM9062</td>
<td>hpIs583</td>
</tr>
</tbody>
</table>

**Calcium imaging:**

<table>
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<tr>
<th>Strain</th>
<th>Insertion</th>
</tr>
</thead>
<tbody>
<tr>
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<td>hpIs459</td>
</tr>
<tr>
<td>ZM9109</td>
<td>hpIs595</td>
</tr>
<tr>
<td>ZM7656</td>
<td>hpIs365</td>
</tr>
<tr>
<td>AQ2953</td>
<td>ljIs131</td>
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</table>

**Strain generated by crossing:**

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<th>Insertion</th>
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<tbody>
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<tr>
<td>ZM8614</td>
<td>hpIs372; hpIs365</td>
</tr>
<tr>
<td>ZM9119</td>
<td>hpIs559; ljIs131</td>
</tr>
<tr>
<td>ZM9120</td>
<td>hpIs583; ljIs131</td>
</tr>
<tr>
<td>ZM9139</td>
<td>unc-13(s69); ljIs131</td>
</tr>
<tr>
<td>ZM9285</td>
<td>hpIs540; hpIs584</td>
</tr>
</tbody>
</table>
3.2. Molecular Biology

Most promoters used in this study were generated by PCR-amplification using C. elegans N2 genomic DNA as a template. Promoters for Pacr-2, Punc-4 and Pacr-5 were cloned from the 3.4 kb, 2.5 kb, and 4.3 kb genomic DNA sequence upstream of the ATG start codon of each gene, respectively. Restriction enzyme sites were added to ends of the promotor sequences for easy substitutions when generating plasmid constructs.

3.2.1. Calcium sensors

Intracellular calcium sensor GCaMP was used in this study to monitor neuronal or muscle activities represented by intracellular calcium levels (Tian et al., 2009). In this study, the GCaMP3 sensor was used for DD and muscle calcium imaging, and the GCaMP6 sensor was used for DA and DB calcium imaging. All GCaMP was fused at the C-terminal with wCherry to allow ratiometric measurement. Constructs that express the GCaMP::wCherry transgene were injected and intracellular fusion protein was expressed in the cytosol of target cells to perform simultaneous imaging of GFP and RFP. This approach was designed to measure the ratio of GFP to RFP as a readout from calcium during the animal’s semi-constrained movement. This step minimizes the motion artifact (Taizo Kawano, unpublished observations).

To monitor the activity of different motor neurons, the following transgenic strains were used: Punc-25-GCaMP3::wCherry (hpIs365) for D motor neurons, Punc-4-GCaMP6::wCherry (hpIs459) for A motor neurons and Pacr-2s-GCaMP6::wCherry (hpIs595) for B motor neurons. ljIs131 (Pmyo-3-GCaMP3::TagRFP) was used for muscle calcium imaging, a gift from Dr. William Schafer (Cambridge).
3.2.2. Photo-inducible flavoprotein for cell ablation

MiniSOG is a flavoprotein, a genetically encoded photosensitizer that yields singlet oxygen upon blue-light activation and effectively kills cells without damaging surrounding tissues (Shu et al., 2011). To optimize ablation efficiency, transgenic animals expressing mitochondria-targeted miniSOG under the neuron specific promoters were generated.

For miniSOG transgene, an intercistronic sequence consisting of a U-rich element and Splice Leader sequence (UrSL) was inserted between the coding sequence of miniSOG and a fluorescent protein reporter to construct a bicistronic expression vector, where miniSOG and the fluorescent report were co-transcribed, spliced, and separately translated and targeted different compartments of the same cell: MiniSOG will be transported into mitochondria, and the fluorescent reporter remains in the cytosol. This system allows us to label cells that express miniSOG and observe their morphology before and after miniSOG manipulation. Two types of the bicistronic expression reporters were used in this study including: 1). wCherry, a C. elegans codon-optimized RFP (a gift from A. Desai, University of California, San Diego), was used for generating behavioral analysis strains; 2) wBFP, a C. elegans codon-optimized BFP (a gift from J. Calarco, Harvard University) was used for calcium imaging strains.

This fluorescent reporter system allowed us to examine the morphology of the neurons for checking the results of ablation. Neurons that were not ablated showed fluorescence in the intracellular space of the soma and the beading-morphology in the axonal region, but not in the nucleus. Ablated neurons showed ruptured axons without
regular puncta and unstructured soma without clear fluorescence-negative nucleus. Occasionally, scattered fluorescence could diffuse into peripheral tissues. Animal with successfully ablated neurons were subjected to recordings for behavior analysis and calcium imaging.

For on-plate behavioral analysis, the following integrated transgenic strains were used: and \textit{Pacr-5-tomm20-miniSOG-UrSL-wCherry (hpIs372)} for B motor neurons, \textit{Punc-4-tomm20-miniSOG-UrSL-wCherry (hpIs371)} for A motor neurons, and \textit{Punc-25-tomm20-miniSOG-UrSL-wCherry (hpIs377)} for D motor neurons.

For in-liquid behavioral analysis and muscle calcium imaging, the following transgenic strains were used: \textit{Pacr-2(s)-tomm20-miniSOG-UrSL-wCherry (hpIs583)} for DA and DB motor neuron ablation, \textit{Pttr-39-tomm20-miniSOG-UrSL-wBfp (hpIs540)} for DD motor neurons ablation and crossed strain \textit{Pacr-2(s)-tomm20-miniSOG-UrSL-wCherry + Pttr-39-tomm20-miniSOG-UrSL-wBfp (hpIs540; hpIs583)} was used for DA, DB and DD neuron ablation.

For calcium imaging upon motor neurons ablation: \textit{hpIs371; hpIs365 and hpIs372; hpIs365} was used for DD imaging upon DA and DB ablation, respectively. \textit{hpIs559} (generated by co-injecting \textit{Pacr-5-tomm20-miniSOG-UrSL-wBFP + Punc-4tomm20-miniSOG-UrSL-wBFP + Pttr-39-tomm20-miniSOG-UrSL-wBFP}); \textit{ljIs131} was used for muscle calcium imaging upon DA, DB and DD ablation; \textit{hpIs583; ljIs131} was used for muscle calcium imaging upon DA and DB ablation.

3.3. Motor neuron ablation

The method for \textit{miniSOG} induced neuron ablation was to apply blue LED over a open,
standard NGM culture plate containing animals of desired age without a lid to, 1) allow proper transmission of LED with full intensity and 2) allow sufficient oxygen. Unless specified otherwise, a 40-minute exposure was used for the blue LED light that we have found to efficiently kill neurons of interest. Animals were typically allowed to recover for several hours to overnight without light.

All strains used for these studies were maintained in darkness unless to be handled for experiments. This was achieved by covering containers for culture plates with the tinfoil. For L1 behavioral analysis and calcium imaging upon neuronal ablation, animals were ablated within 1 hour post-hatching from the egg. Briefly, three-fold stage eggs were hand-picked and placed on NGM plate. 1 hour later, all hatched L1s were transferred onto another plate for blue LED treatment. After 40 minutes of blue light illumination, the plate was kept in darkness for about 2-3 hours to let animals recover. These animals were then subjected to recording (on-plate/in-liquid behavioral analysis or calcium imaging). After recording, all animals were transferred back to the NGM plate to allow them develop so that we could check whether the neuronal ablation was complete later. For L4 behavioral analysis, L2/L3 transgenic animals were exposed under blue LED light for 40 minutes, and allowed recovery in darkness overnight. Early L4 animals were selected for behavioral recording and rescued afterwards to examine the ablation efficiency. All data that were analyzed and presented in this thesis were from animals where complete neuronal ablation was confirmed.

3.4. Behavioral analysis

3.4.1. The on-plate locomotion analysis
Animals for the behavior assay were transferred to a NGM plate seeded with a very thin layer of OP50 (recording plate) and allowed 1 minute of habituation before recording. Data pooled and compared were generated from animals recorded using the same recording plate. The images were captured on a Zeiss Axioskop 2 Plus microscope equipped with an ASI MS-40000 motorized stage and a CCD camera (Hamamatsu Orca-R2). Image sequences were sampled at 100 ms exposure or 10fps for 3 minutes by the in-house developed software plug-in through Micromanager and ImageJ. During the recording, the program controls an automated tracking program that detects the centroid of the animal, tracts and re-centers the animals. The on-image X, Y coordinates of the centroid of the animal and the X, Y coordinates of the motorized stage were recorded for each frame to perform velocity calculation.

The post-imaging analysis was performed with in-house developed Image J plug-ins (Kawano et al., 2011; Kawano, unpublished). Firstly, the outline of the animal was detected and the centerline of the animal was extracted. The “head” and “tail” was then identified, between which the centerline were divided into 33 equally spaced segments. The angle between each segment and the extended line from the adjacent anterior segment was calculated to represent the curvature of that segment of the animal. Images of animals that touched the edge of recording field and that crossed over themselves were not processed. The absolute position of the centroid points was determined based on the coordinates of its position in the field-of-view and the stage, and was used to calculate the velocity and direction of the animal’s movement. The velocity was calculated by dividing the pixel displacements of the centroid by the exposure time per frame (100 ms). The movement direction was defined as the following: 1) forward movement as when the
displacement was towards head at bigger than 1 pixels per second; 2) backward movement as when the displacement was towards tail at bigger than 1 pixels per second and 3) pausing as the velocity between −1 (− was defined as movement towards the tail) and +1 (+ was defined as movement towards the head) pixel per second.

The output of the primary data was then used for quantification analyses using an R program based script written by Michelle Po, a former postdoctoral fellow (Gao et al., 2015). The program automatically sorted the data set from a group of recordings (N=10 for each condition), and output pooled data from all the results including: 1) the duration of forward and backward movement that lasted equal to or more than 3 frames, or 0.3 second (we defined that a period of directional movement should have more than two consecutive frames where the centroid moved in the same direction); 2) velocity histogram; 3) total percentage of time that all animals from the group spent in forward or backward motion (movement >1 pixel) or pausing (movement <1 pixel); and 4) Initiation frequency of the forward and backward movement.

3.4.2. The in-liquid swimming analysis

The in-liquid behavior was recorded for worms placed in a thin layer of 30% dextran solution sandwiched between two glass slides. Newly hatched L1 was ablated following the method described above (Section 3.3.1). 2 uL droplets of 30% dextran solution (Catalog 9004-54-0, Sigma-Aldrich) was placed on a glass slide. Two strips of polycarbonate spacer of 25.4 microns thickness (Catalog # 9513K12 from McMaster-Carr, a gift from Dr. Aravithan Samuel) were positioned on two sides of the droplet, and were apart by a distance shorter than the length of a coverslip. Once an animal was
placed in the dextran solution, one cover slip was placed on top of the droplet with the spacer supporting its weight.

Images were captured on a Zeiss Axioskop 2 Plus microscope under dark-field fluorescence recording setup, equipped with an ASI MS-40000 motorized stage. Green light illumination was used to visualize the wCherry reporter in the miniSOG transgenic animals. Using the VNC motor neuron specific fluorescent marker (DA, DB and/or DD), we could determine the ventral and dorsal side of the animals in each recording. Image capture was done using ImageJ, each recording was performed at 100 ms exposure (10 fps) and lasted 3 minute, with N=10 for each ablated and control group. The calculation of the biased dorsal/ventral bending time and frequency was manually analyzed by visual examination of these videos.

3.5. Calcium Imaging and data analysis

3.5.1. Real-time calcium imaging

To record calcium transient in motor neurons and muscles, L1 C. elegans were placed on top of a wet, freshly made 2% agarose pad with a small drop of the M9 buffer to allow movements. L1s selected for imaging were within 5 hours post hatching from embryos.

Images were captured on a Zeiss Axioskop 2 Plus equipped with the ASI MS-40000 motorized stage, a Dual-view beam splitter (Photometrics, Tucson, AZ) and a CCD camera (Hamamatsu Orca-R2). The fluorescence excitation light source from X-CITE (EXFO Photonic Solution Inc.) was adjusted accordingly to avoid saturation of GCaMP protein or wCherry reporter. The 4x4 binned images were obtained at 50 ms exposure or 20fps for all recordings. The fluorescent images were split by Dual-View
beam splitter with a GFP/RFP filter set onto the CCD camera operated by Micromanager.

3.5.2. Post-imaging analysis

3.5.2.1. Motor neuron calcium imaging analysis

For motor neuron imaging, regions of interest (ROIs) containing the soma or axonal regions of neurons of interest were defined, and the GFP and RFP fluorescence intensities was measured using in-house developed ImageJ plug-ins (Kawano et al., 2011). The GFP to RFP ratio was used as the parameter to represent calcium transient to reduce motion artifacts. Two additional parameters were calculated for motor neuron calcium imaging: velocity and bending angle. The calculation of relative movement velocity movement was done with a R based script (Kawano et al., 2011; Gao et al. 2015). The bending angle of different ROI was defined to represent contraction and relaxation state of dorsal/ventral body at the ROI (Fig 2-11). For each ROI, the coordinates of the absolute position in each frame of recording were extracted. The coordinates of three consecutive ROIs in anterior to posterior sequence were used to calculate the angle of the ROI in the middle (Fig 2-11, point B). Two vectors B→A and C→B formed an angle and the vector’s coordinates were used to calculate the value of the angle with a MATLAB (MathWorks) script that I wrote (Guan S., unpublished). An angle > 180 degrees was defined as dorsal contraction (or ventral relaxation), while the angle < 180 degree was defined as dorsal relaxation (or ventral contraction). Cross-correlation analysis, performed using the MATLAB, calculated the phase-lag between: 1) activities of anterior-posterior pair of DD motor neuron axons; 2) DD axon activity vs. angle of axonal region; and 3) DA or DB motor neuron activity vs. angle of DA or DB
soma region, respectively.

3.5.2.2. Muscle calcium imaging analysis

For muscle imaging, a MATLAB script (Lu Y., unpublished) was used to measure the dorsal and ventral muscle GFP/RFP ratio, respectively. The “spine” of the entire animal was extracted by centerline detection algorithm through following steps. First, the boundary of an animal was detected through muscle RFP signals. Then, the head versus tail, and the ventral versus drosal side were manually defined by the user. Each dorsal and ventral boundary was divided into 99 equally spaced points (101 points in total including the head and tail). Each pair of corresponding points on the dorsal and the ventral side was connected, and the midpoint of each line was determined. The spine was generated by connecting midpoints, starting and ending with the head and the tail point, respectively. For each segment between the dorsal/ventral boundary and the spine, the GFP and RFP signal of the muscle was measured separately and the ratio of their means was used to represent the calcium transient of dorsal/ventral muscle. The curvatures of all the points on the spine were plotted against of the dorsal/ventral muscle calcium signals. Images of animals that crossed over themselves were clipped and not processed.
Figure 2-11. Calculation of bending angle at Region of Interests (ROIs)
References


Richmond, J. (2009). Dissecting and recording from the C. Elegans neuromuscular junction. Journal of visualized experiments : JoVE.


